Cell-autonomous and non-cell autonomous protection of DNAJB6 in Huntington's disease

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CHAPTER 1

OUTLINE OF THE THESIS

Several neurodegenerative diseases are initiated by protein aggregation in neurons and are associated with a multitude of responses in non-neuronal cells in the brain, in particular glial cells like astrocytes.

These non-neuronal responses have repeatedly been suggested to play a disease-modulating role, but how these may be exploited to delay the progression of neurodegeneration has remained unclear.

Interestingly, one of the molecular changes that astrocytes undergo includes the upregulation of certain Heat Shock Proteins (HSPs or chaperones) that are classically considered to maintain protein homeostasis and protect cells from proteotoxic stress.

The aim of my research project was to explore if and how a specific HSP (DNAJB6) expressed either exclusively in neurons or exclusively in astrocytes can provide *in vivo* protection against protein aggregation and toxicity of Polyglutamine (PolyQ) huntingtin, the mutant protein associated with Huntington's disease.

Chapter 2 of this Thesis provides a general overview on neurodegenerative diseases, including PolyQ diseases that are characterized by the aggregation of mutant proteins (e.g. huntingtin), neuronal degeneration and astrocyte reactivity. The role of the different HSP families and how they contribute to the protein quality control in the cells are presented. I explain the mechanisms underlying protein aggregation, why protein aggregates are toxic for cells, and why neurons are particularly vulnerable. I also provide an overview of the prion-like processes observed for different disease-causing aggregate species. Next, the roles that astrocytes are thought to play in the healthy brain and in the brain affected by neurodegenerative diseases are presented. I focus on how the astrocytes react to protein aggregation and protein aggregates and how they differ in this when compared to neurons. Moreover, it is discussed how astrocytes may intervene in the process of prion-like spreading of aggregates. Next, a systematic review is provided on what is known about expression of HSPs in astrocytes in neurodegenerative diseases, using data from patients and animal models. Based on all this information our hypothesis is presented in which we propose that the expression of specific chaperones in astrocytes during disease might be not only a "marker of stress" of reactive astrocytes, but instead an important mechanism of non-cell autonomous protection mediated by astrocytes towards neurons.

In **Chapter 3**, I next describe the *in vivo D.melanogaster* models that was generated for this research project. To fully explore whether and how the neuronal or astrocytic expression of HSPs contributes to neuroprotection in neurodegenerative diseases, I generated *D.melanogaster* models that exclusively express a mutant toxic protein in neurons, whilst co-expressing a protective chaperone either in the same neurons or in astrocytes. To do so, we used two different binary expression systems (GAL4-UAS and LexA-LexO) combined with cell-type specific promoters to express the transgenes in all neurons (using the driver *elav*), in all glial cells (using *repo*), or specifically in astrocytes (using *alrm*). Moreover, *D.melanogaster* models expressing the transgenes in ommatidia cells (using the driver *gmr*) have been also settled to perform other additional

experiments. Next, I present the data from these *D.melanogaster* models that exclusively express a mutant toxic PolyQ protein in neurons, whilst co-expressing a protective chaperone (DNAJB6) either in the same neurons (to study cell autonomous effects, **Chapter 4**) or in astrocytes (to study non-cell autonomous effects, **Chapter 5**).

Our data show that DNAJB6 can provide cell autonomous protection against PolyQ-mediated neurodegeneration in *D.melanogaster*, which is associated with a reduction in the PolyQ-protein aggregate load in the fly brains (**Chapter 4**). Intriguingly, the exclusive expression of DNAJB6 in astrocytes also provides non-cell autonomous protection against progressive neuronal degeneration and prolongs organismal lifespan (**Chapter 5**). However, this is not accompanied by a reduction in the PolyQ-HTT aggregate load in the fly brains. Rather, under these conditions, a high fraction of astrocytes now contains neuronal-derived PolyQ-HTT aggregates, in line with the suggestion that astrocytes might take up PolyQ-HTT aggregates species to halt neuron-to-neuron spreading, a capacity that is enhanced by DNAJB6 expression. Therefore, our data indicate that astrocytes play a role in the prion-like processes of PolyQ diseases and that the overexpression of specific protective HSPs - such as DNAJB6 - can boost the non-cell autonomous functions of astrocytes in protecting neurons (**Chapter 5**).

In **Chapter 6**, I discuss on how DNAJB6 can lead to neuroprotection in a non-cell autonomous manner. I discuss the mechanisms of prion-like propagation in PolyQ diseases and the possible role of astrocytes in each of these processes. Moreover, I provide ideas on whether and how astrocytes could be used as target for therapy, by boosting their capacity to handle toxic aggregates through the potentiation of their chaperonome and therefore by potentiating their non-cell autonomous protective functions.

CHAPTER 2

INTRODUCTION

Matteo Bason and Harm H. Kampinga* (in preparation for review)

1. Neurodegenerative Diseases: a global disease emergency

Neurodegenerative Diseases (NDs) are considered by the World Health Organization to be part of the large group of neurological disorders affecting the brain and can be described as a degeneration of a specific population of neurons in the human nervous system. NDs selectively target specific population of neurons and neuronal circuits leading to the progressive failure of defined brain systems. In NDs patients, the degeneration of such neurons and circuits determines a different spectrum of symptoms, which reflect the loss of that particular population of neurons and their normal function in the central nervous system. Loss of neurons cause distinctive symptoms like motor disturbances such as chorea in Huntington's disease (HD) or bradykinesia in Parkinson's disease (PD), or loss of cognitive function such as dementia in Alzheimer's disease (AD). The disease typically initiates in specific brain areas, but progressively affects other regions of the brain with manifestations of new symptoms or the worsening of early ones.

NDs are generally a chronic condition, characterized by a slow progression over time, which can extend for decades. Aging is strongly associated with the decline of brain functionality and the risk of developing sporadic forms of NDs, like AD and PD, rises sharply with age after 60, although some genetic forms of NDs, like HD, may have an early-age onset (Wolfe, 2018; section 2.3).

Given the increasing age of the world population and the absence of therapies against these chronic, highly debilitating and care-costing diseases, the World Health Organization considers brain disorders, AD and PD in particular, as leading contributors to the global disease burden. For instance, Alzheimer's Disease International organization estimates that 50 million people worldwide are living with dementia in 2018 (AD accounts for an estimated 60-80 percent of these cases) (Alzheimer's Disease International, 2018). This number is projected to increase to about 150 million by 2050, as the population ages (Figure 1).

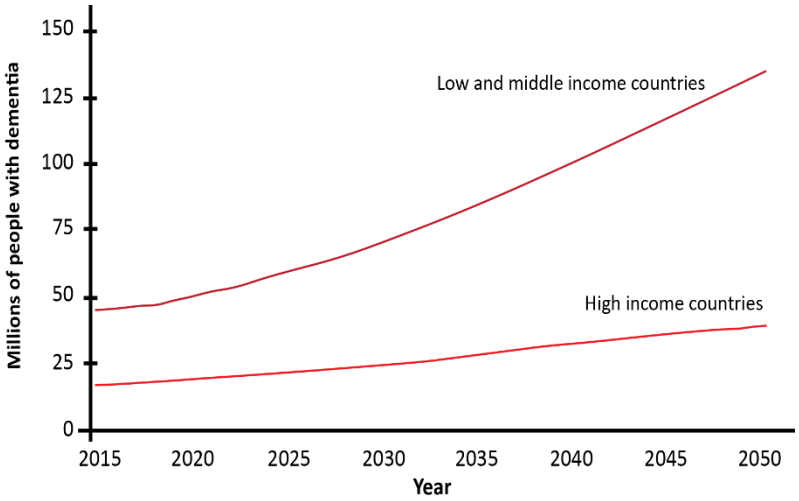

***Figure 1*: Number of people with dementia in low and middle income countries compared to high income countries.** *The World Alzheimer Report 2015 indicates 9.9 million new cases of dementia each year worldwide (one new case each 3.2 seconds). In the future, as the health care will improve, the consequent global demographic ageing will cause a progressive increase of dementia cases, especially in low- and middle-income countries (Source of the data from World Alzheimer Report 2015, https://www.alz.co.uk/research/statistics)*

Current treatments for all NDs are only symptomatic (e.g. Levodopa to control motor symptoms in PD or anti-cholinesterase agents against dementia in AD) (Wolfe, 2018). A better understanding of pathogenic mechanisms of NDs is essential to develop effective therapeutic strategies. Although

each ND shows a different biology, focusing on the common features of these diseases may provide important information about the key factors of neuronal degeneration.

2. Protein aggregation as common clue in neurodegenerative diseases

Although these pathologies have different characteristics and causes, the main common hallmark in the post-mortem brains of patients affected by different NDs is the presence of protein aggregates in the degenerated tissue. This provided an important clue to neurobiologists: the process of protein aggregation might be toxic for cells and somehow causative for NDs (Ross et al., 2004; Eisele et al., 2015; Wolfe, 2018).

Yet, there is an active discussion related to the type of aggregates responsible for (neuro) toxicity, with some investigators even arguing that aggregates may not be toxic at all and just a by-product of the degenerative process (Eisele et al., 2015).

Here I want to argue that the (process of) formation of aggregates is driving the disease because:

1. In all heritable, early onset forms of these diseases, the mutated genes encode proteins with reduced stability and high aggregation probability (section 2.3).
2. Mutations in molecular chaperones (chaperonopathies) and other protein quality control components (UPS or autophagy components) with a physiological role to prevent protein aggregation (see next sections), also lead to neuro and (cardio) muscular diseases associated with protein aggregation (sections 2.2 and 2.3).
3. In several studies using experimental models of these diseases, modulation of components of the protein quality control network were found to delay disease onset and progression (section 2.1).
4. Addition of *in vitro* generated protein fibrils can induce degeneration in several experimental models (section 2.6).

Therefore, an extensive part of the research in the field focused on understanding why and how proteins form aggregates and by which mechanisms aggregates may contribute to cell degeneration and death. In the neurodegenerative process, degeneration and death of neurons appear to primarily occur during the late stage of disease, preceded by functional (e.g. electrophysiological deficits, change in gene expression) and morphological (e.g. loss of synaptic connections and axon retraction) alterations (section 2.4).

Protein aggregation diseases (even the genetic forms) are typically late-onset. This suggests that in young individuals, the protein quality control (PQC) systems of the cells - the ensemble of systems that control protein folding and refolding, protein transport, protein complex (re)modelling and protein degradation via the ubiquitin-proteasome system (UPS) or the autophagy pathways - is capable to maintain protein homeostasis and hence inhibits the initiation of the toxic aggregation process. We here define protein homeostasis as the physiological balance between protein production and protein quality control. Protein aggregation in diseases may be initiated as a result of an age-related increases in the burden of unfolded or misfolded proteins and /or and age-related

decline in the functioning of the PQC network, (i.e. when protein homeostasis is perturbed). Aggregate-associated NDs can be then defined as age-related proteinopathies, in which protein aggregation is the main cell autonomous insult capable to trigger the neuronal degeneration (section 2.2).

In this Chapter, I will first discuss the PQC and the role of the heat shock proteins (HSPs), the key regulators of the cellular PQC and main contributors to the protein homeostasis: HSPs represent the first line of defense against protein aggregation, primarily acting in a cell-autonomous manner. Next, I will discuss NDs-associated aggregates and their toxicity in cells and the factors that trigger aggregation and overwhelm the cell autonomous protective strategies.

2.1: Protein quality control: HSPs and cellular strategies against aggregation

A protein is translated on ribosomes as a chain of amino acids, which must fold in a specific three-dimensional structure called "native state" to perform its biological functions. Although proteins may reach the native state guided by the primacy of their amino acid sequence (as postulated by Anfinsen's dogma) (Anfinsen, 1973), protein folding in cells requires assistance not least because protein aggregation must be prevented. To prevent that native polypeptides, but also unfolded or misfolded protein species aggregate, all living systems have a PQC network in which HSPs (also called molecular chaperones) mediate multiple key processes to maintain the protein homeostasis in the normal cellular environment (Hartl et al., 2011). The human genome encodes more than 100 different HSPs which are grouped in different families: HSPH (HSP110), HSPC (HSP90), HSPA (HSP70), DNAJ (HSP40), HSPB (small HSPs) and the chaperonins HSPD-E (HSP60-HSP10) and CCT (TRiC). Several regulatory co-factors, such as the members of the BAG proteins family, can also be included in this network (Kampinga et al., 2009).

In a recent review, Kakkar and colleagues (Kakkar et al., 2014) summarized the literature on findings showing that HSPs can prevent protein aggregation in different NDs. This analysis also revealed that for each of the different types of proteins in the different neurodegenerative disease, different members of the HSPs family seemed required, implying that although aggregation drives these diseases, the types of aggregates or pathways of aggregation may substantially differ for the various disease-associated proteins.

However, how do HSPs generally contribute to PQC and aggregate prevention in cells? HSPs guide all the proteins in the cells from production to degradation, without being directly involved in their biological functionality, and show a great diversity in transcriptional regulation and functional capacity, which both depend on the "client" that they are processing and the conditions which the cells are coping with (Morimoto et al., 2008; Hartl et al., 2011). Despite their name, many HSPs are constitutively expressed in normal growing conditions and are essential for the cell viability (Morimoto et al., 2008). The constitutive HSPs participate in the *de novo* folding of proteins, in protein transport in the cellular compartments, in the (dis)assembly of protein complexes, in the protein degradation, and generally act as a buffer to counterbalance the natural tendency of proteins to unfold, misfold or aggregate (Hartl et al., 2011).

During co-translational folding, ribosome-associated HSPs (e.g. specialized HSPAs/HSP70 and DNAJs/HSP40) provide initial folding assistance to the nascent amino acidic chain (Kampinga et al., 2010; Hartl et al., 2011). Subsequently, the client is folded by the classical HSPA/HSP70 machinery (Kampinga et al., 2010). Here, partners of HSPA/HSP70 are DNAJs/HSP40 and nucleotide-exchange factors (NEFs), which regulate the interaction between the client and HSPA, by affecting the HSPA affinity for adenine nucleotides (ATP or ADP) (Kampinga et al., 2010).

A canonical HSPA machinery acts through the following steps (Kampinga et al., 2010; Dekker et al., 2015; Zuiderweg et al., 2017; Figure 2):

1) The J protein binds the non-native client protein and interacts with HSPA-ATP through its J domain. By this initial binding, the J protein prevents the possible aggregation of the non-native client (Dekker et al., 2015). Also, small HSPs (sHSPs) can act as an entry step of clients to the Hsp70 machine (Boncoraglio et al., 2012; Garrido et al., 2012). These sHSPs bind unfolded or misfolded protein to form chaperone-client complexes that prevent a (more) irreversible aggregate formation, keep substrate competent for processing by the HSPA/HSP70 machinery(Boncoraglio et al., 2012; Garrido et al., 2012; Carra et al., 2017; Haslbeck et al., 2019).

2) The client interacts with the peptide binding site of HSPA and, together with the J domain, stimulates HSPA to hydrolyze the ATP. This drives a conformational change in HSPA that stabilizes its interaction with the client by closing the peptide binding domain and causes DNAJ to leave the complex.

3) The NEFs (such as HSPSH/HSP110, HSPBP1 and BAG family proteins) bind HSPA-ADP and mediate the ADP-ATP exchange, reverting HSPAs to their “open” conformation and leading to the client release (Kampinga et al., 2010; Rampelt and Bukau, 2011; Bracher et al., 2015). In this phase, the client is released and may have reached its native, functional conformation. If folding is not completed after the release, the client will re-enter the cycle and may get to their final state by reiterative cycles of binding and release. Clients that cannot be completely folded by the HSPA/HSP70 machinery are transferred to or handled independently by the HSPC/HSP90 system, via the HSP-organizing protein (HOP) mediation (Young et al., 2001), or by the chaperonins (GroEL/GroES and TRiC) (Spiess et al., 2004). Clients that cannot be refolded at all, can be transferred to degradation machines (see below).

Acute proteotoxic stress conditions such as heat shock, oxidizing agents (e.g. reactive oxygen species (ROS)) and any other environmental factor can cause many proteins to become unfolded or misfolded and thus imbalance the protein homeostasis of the cells, with the risk of aggregate formation (sections 2.2.-2.3). Although the constitutively expressed HSPs might still assist in the refolding of these proteins, the cell also activates several stress responses pathways that upregulate selected HSPs via the induction of transcriptional programs in different cell compartments to rebalance protein homeostasis. Yet, only some HSPs genes are constitutively expressed and upregulated under stress or are expressed only under stress conditions; in fact most are not upregulated by proteotoxic stress and likely serve in other aspects of ensuring protein homeostasis

(Morimoto et al., 2008; Hageman et al., 2009; Mahat et al., 2016; Solis et al., 2016; Neueder et al., 2017).

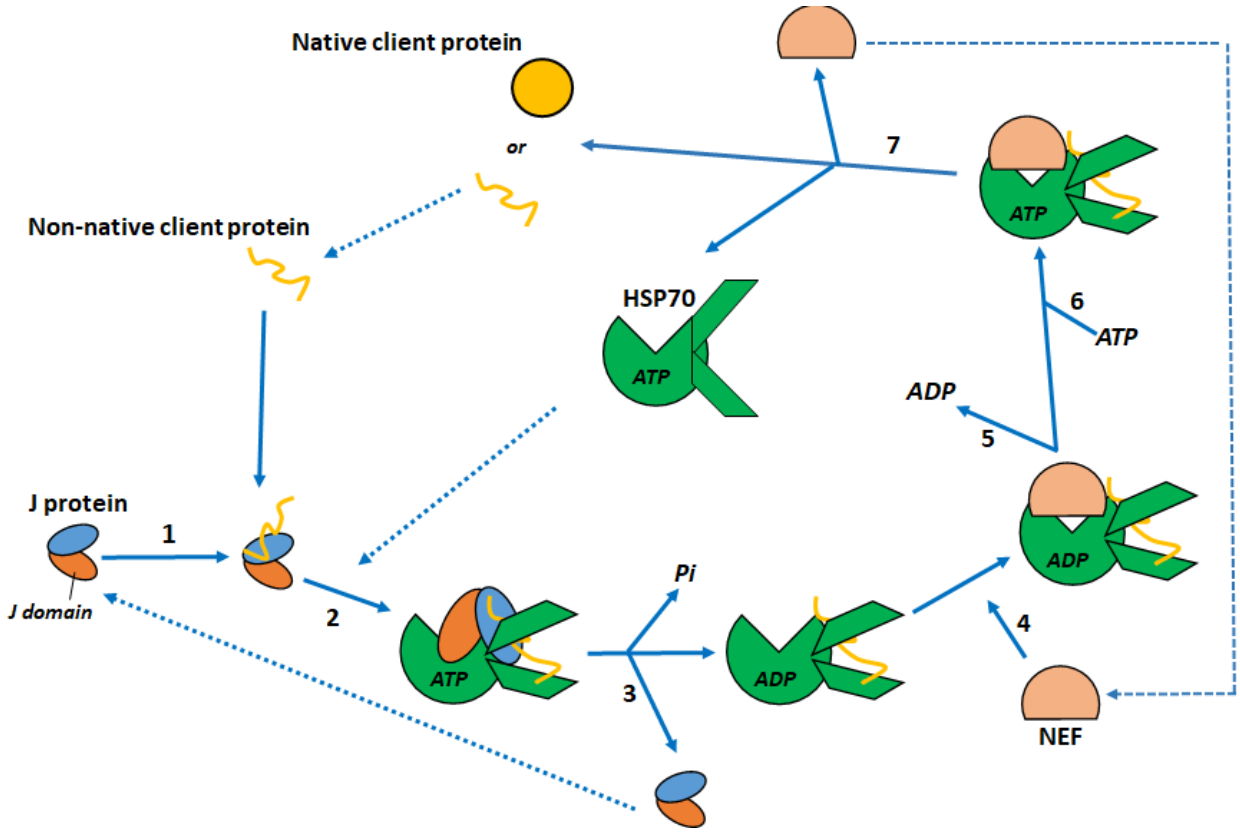

Figure 2: Canonical model of action of HSPA / HSP70 machinery in protein folding. *(Based on the model from: Kampinga et al., 2010 - The HSP70 chaperone machinery: J proteins as drivers of functional specificity).*

The cytosolic stress response is mainly controlled by the heat shock transcription factor 1 (HSF-1) (Morimoto, 2011), which up-regulates several HSPs genes including members of small HSPs like *HSPB1* (*HSP27*) and *HSPB5* (*αB crystallin*), DNAJ proteins like *DNAJB1* (HSP40) and HSPA members like *HSPA1A*, *HSPA6* and *HSPA8* (Kampinga et al., 2009; Hageman et al., 2010). The HSF-1 dependent cytosolic cell response is interconnected with the unfolded protein response (UPR) pathways occurring in the endoplasmic reticulum (UPR^{ER}) (Walter et al., 2011) and in the mitochondria (UPR^{MT}) (Haynes et al., 2010). The accumulation of stress-denatured proteins in the lumen of these organelles is one of the main activation factors for both UPR^{ER} and UPR^{MT}. From the ER, folded proteins are transported to the Golgi apparatus for further processing, whereas improperly folded proteins are degraded via proteasomes after retro-translocation in the cytosol, in a process called ER-associated degradation (ERAD). Under proteotoxic stress conditions, stress-denatured proteins are accumulated in the ER and are sensed by three different signal transducers (ATF6, PERK and IRE1) that activate the UPR^{ER} pathway (Walter et al., 2011). The UPR^{ER} positively regulates the expression of numerous genes, which down-tune the overall protein translation and encode for HSPs that increase the ER protein-folding capacity and the protein degradation via ERAD, Ubiquitin-Proteasome System (UPS) and lysosome-mediated pathways (Walter et al., 2011). In mitochondria, proteotoxic stress conditions, in particular aging-related factors (e.g. respiratory chain dysfunction

and increased ROS in senescent mitochondria), concur to the accumulation of stress-denatured proteins in the mitochondrial matrix or intermembrane space. The consequent UPR^{MT} activation regulates a broad transcriptional program which includes genes encoding for mitochondrial chaperones that assist the organelle in protein refolding and quality control during the proteotoxic stress (Haynes et al., 2010).

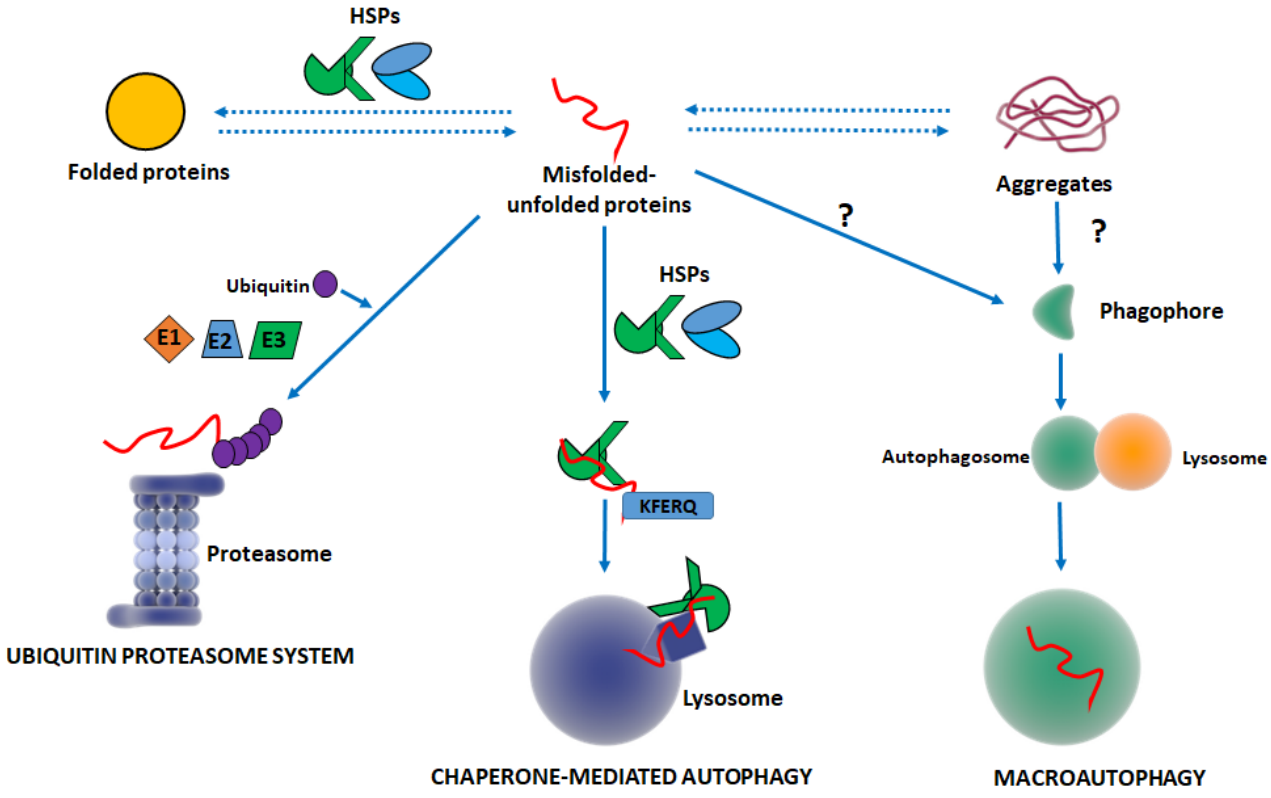

Figure 3: Protein degradation pathways in cells*. (Based on the model in Chiecanover et al., 2015 - Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies).*

As stated above in case of failure of protein folding, the chaperone machineries can re-route the client for its degradation via: 1) the ubiquitin proteasome system (UPS) (Ciechanover et al., 2015) 2) the macroautophagy pathway (Tyedmers et al., 2010; Feng et al., 2014; Ciechanover et al., 2015) or 3) the chaperone-mediated autophagy (CMA) (Chiecanover et al., 2015; Kaushik and Cuervo 2018). As in the folding process, the pathways involved in protein degradation depend on the client and the cellular conditions (e.g. starvation and amino acidic need, accumulation of unfolded or misfolded proteins during proteotoxic stress) (Tyedmers et al., 2010; Ciechanover et al., 2015) (Figure 3).

The prime pathway for most unfolded or misfolded proteins generated in the various cell compartments is the UPS, a selective proteolytic system for soluble single proteins, in which the conjugation of the client to ubiquitin (Ub) determines its degradation by the proteasome (Herskho et al., 1998). The process of ubiquitination is mediated by an enzymatic cascade composed by three main types of enzymes: the E1 Ub-activating enzymes, the E2 Ub-conjugating enzymes and, the E3

Ub-ligases that selectively recognize and (poly) ubiquitinate the client (Herskho et al., 1998). The properly ubiquitinated client is delivered to the proteasome. Here it is de-ubiquitinated, unfolded, inserted into the "chamber" of the proteasome and progressively cleaved into shorter small peptides. HSPs assist the UPS in the recognition of unfolded and misfolded proteins, in their ubiquitination by E3 ligases and finally in their delivery into the proteasome for the cleavage into small peptides, that can be further processes into single amino acids via aminopeptidases (Ravid et al., 2008; Cheicanover et al., 2015).

The interplay between HSPs and the proteasomal degradation is, however, far from understood: co-chaperones of the HSPA/HSP70 machinery such as DNAJB2 (Gao et al., 2011) and BAG1 (Luders et al., 2000) can interact with subunits of the proteasome and some E3 ligases can affect the function of the HSPA/HSP70 cycle (Rosser et al., 2007). This suggests that certain HSPs are part of the mechanism that targets proteins to proteasomal degradation, but how triaging between refolding and degradation occurs is still unknown.

In macroautophagy, the client intended for the degradation (e.g. a portion of the cytoplasm, unfolded and misfolded proteins, protein aggregates or even entire organelles) is collected into the autophagosome, a double-membraned vesicle, which subsequently fuses with a lysosome, causing the final degradation of the cargo by the lysosomal proteases (Nakatogawa et al., 2009). Each step of macroautophagy, such as the formation of the autophagosome and its fusion with the lysosome, is narrowly controlled by and involves several protein adaptors and regulators (Nakatogawa et al., 2009). HSPs can participate to the recognition of the cargo and its delivery into the autophagosome: a well-studied example of this is a process called "BAG-instructed proteasomal to autophagosomal switch and sorting" (BIPASS), a pathway that involves the co-chaperone BAG3 in promoting the degradation of the client to the autophagic pathway (Carra et al., 2008; Gamerdinger et al., 2011; Minoia et al., 2014).

CMA is a specific form of autophagy in which specific misfolded proteins that expose the amino acidic motif KFERQ (a motif found in about 30% of cytosolic proteins and normally buried in the native state), are selectively recognized by HSPA8/HSC70 and other co-chaperones. The client is delivered on the lysosomal membrane where it is unfolded and translocated into the lumen and degraded by lysosomal proteases into amino acids (Kaushik et al., 2018).

HSPs represent the first line of defense against protein aggregation and, coupled with the described mechanisms of protein degradation, contribute to maintain the protein homeostasis in the cell. However, if aggregation could not be prevented, cells have additional means to counteract aggregate toxicity by sequestering the misfolded or aggregated proteins into inclusions to prevent their toxicity. Such regulated deposition of endangered protein species in specific cellular deposit sites is a key strategy of defense against protein aggregation observed throughout the evolutionary trees (i.e. from bacteria to yeasts and mammalian cells) (Tyedmers et al., 2010). The type of deposition may differ depending on the stress conditions, the type of aggregating proteins and the cellular compartment. Particularly, several regulated and membrane-free deposition sites of aggregates have been observed in yeasts (Kaganovich et al., 2008; Miller et al., 2015a; Miller et al., 2015b; Rabouille and Alberti; 2017), including:

1) The juxtanuclear JunQs (Kaganovich et al., 2008) and intranuclear (INQs) (Miller et al., 2015b) quality-control compartments, in which ubiquitylated proteins are transiently stored when the degradative capacity of the UPS is saturated.
2) The insoluble protein deposits (IPoDs) (Kaganovich et al., 2008), in which aggregating proteins are accumulated in a seemingly more permanent manner (i.e., not destined to further processing or degradation via UPS).

In addition, a transient form of regulated aggregate deposition, called aggresomes, has been observed in mammalian cells (Johnston et al., 1998; Kopito et al., 2000; Garcia-Mata et al., 2002): aggregates formed at the periphery of the cell are moved along microtubules to the perinuclear site of the microtubule-organizing center (MTOC) (Garcia-Mata et al., 1999). This movement is mediated by the activity of the motor protein dynein and adaptor proteins, like histone deacetylase 6 (HDAC6), which are capable to recognize the ubiquitylated cargos (Kawaguchi et al., 2003). In how far aggresomes can be directly related to the IPoDs and/or JunQs is yet unclear. The INQs found in yeasts may be comparable to nucleolar sequestration of misfolded proteins in mammalian cells for nuclear proteins (Nollen et al.,2001; Park et al., 2013) that may also serve to transiently store misfolded cytosolic proteins, for further refolding (Nollen et al., 2001) or degradation (Park et al., 2013).

Interestingly, evidence has also shown that a process of disaggregation may occur that is conserved from bacteria to human, albeit that the players involved seem to differ (Mogk et al., 2018). Disaggregation is essentially characterized by the recognition of the aggregate by sets of HSPs that actively participate to the one-by-one extraction of misfolded polypeptides. The extracted proteins are then likely destined to subsequent HSPs-regulated processes of refolding or degradation. Disaggregation in yeasts is mediated by the yeast-specific chaperone HSP104, a member of the AAA+ ATPases associated with diverse cellular activities, in cooperation with the yeast HSP70 chaperone systems (Sanchez and Lindquist 1990; Parsell et al., 1994; Lindquist et al., 1996; Glover and Lindquist 1998). Importantly HSP104 homologs are absent from metazoan (with the exception of mitochondria); disaggregation in mammalian cells, however, does occurs and it is mainly mediated by HSPA/HSP70 (i.e. HSPA8 and HSPA1A in humans) which is assisted by specific set of co-chaperones that empowers HSPA/HSP70 to exhibit a potent, standalone disaggregation activity. These co-chaperones include members of the HSP110 family (HSPH1 in humans) and DNAJs (e.g. DNAJA2 and DNAJB1 in humans) (Nillegoda et al., 2015; Mogk et al., 2018). A proposed mechanism to describe the process of disaggregation is the “pulling model”, in which HSPA uses the energy of DNAJ-stimulated ATP hydrolysis to lock the substrate at the aggregate surface and apply force to “extract” it. sHSPs are thought to facilitate this “extraction” process if present during the formation of the aggregate: during this process, they bind to the aggregating substrates hereby changing the structure of the aggregates such that they remain in a (more) disaggregation-competent form (Nillegoda et al., 2015; Mogk et al., 2018).

Finally, another important strategy against protein aggregation, which is observed in bacteria (Lindner et al., 2008; Winkler et al., 2010), yeasts (Aguilaniu et al., 2003) and mammalian cells (Rujano et al., 2006; Fuentealba et al., 2008), is the asymmetric partitioning of aggregates during cell division. It has been shown that the mother cell in yeast and non-stem cells in metazoan may

retain most of the aggregate species during mitosis, benefiting the daughter cell with a lower aggregate load ("dilution effect"). Importantly, the almost total absence of mitosis in adult human neurons imply that these post-mitotic cells are not capable of this form of aggregates clearance, partially explaining the intrinsic vulnerability of neurons to protein aggregation and toxicity (section 2.5).

The presence of protein aggregates in the brain of NDs patients indicates that the previously described PQC pathways and strategies have failed to maintain the protein homeostasis. What are then the main contribution factors of protein aggregation? What are the main types of protein aggregates found in the brain of NDs patients? Which aggregates are characteristic for each specific NDs?

2.2. Factors leading to age-related protein aggregation

Aging is the main factor that enhances the probability of protein aggregation (Koga et al., 2011; Higuchi-Sanabria and Dillin, 2018). With age, a general decline in the capacity of the PQC and degradation pathways have been reported (Figure 4 -green line; Higuchi-Sanabria and Dillin, 2018). Inversely, the protein damage burden may increase due to many factors including accumulated oxidative damage, molecular errors during protein translation with mis-incorporation of amino acids or during the assembly of protein complex, and accumulation of somatic genetic alteration. As a consequence, protein homeostasis collapses when the burden exceeds the PQC capacity (Balch et al., 2008; Bhreme et al., 2014; Kampinga and Bergink, 2016;).

As illustrated in the hypothetical model in Figure 4, this would lead to the onset of sporadic forms of NDs, such as AD or PD (Figure 4 - red line). Support for such a model is furthermore provided by the genetic forms of NDs of AD (e.g. due to mutations in amyloid precursor protein or tau; Bird et al., 2012) or PD (due to mutations in α-syn) that lead to an elevated aggregation propensity of the affected proteins. Such evidence is even better illustrated by purely genetic NDs, like PolyQ diseases, where patients express an aggregation-prone protein from birth, yet are generally unaffected till mid-life (Figure 4 - blue line) due to an early collapse in protein homeostasis (Zuccato et al., 2010). Additional evidence for this model comes from diseases due to mutations in chaperones (so-called chaperonopathies; Macario et al., 2002, Kakkar et al., 2014) or other components of the PQC system (e.g. autophagy; Ciechanover, 2015), that are also often associated with protein aggregation (Figure 4-yellow line, and sections 2.3 and 2.4).

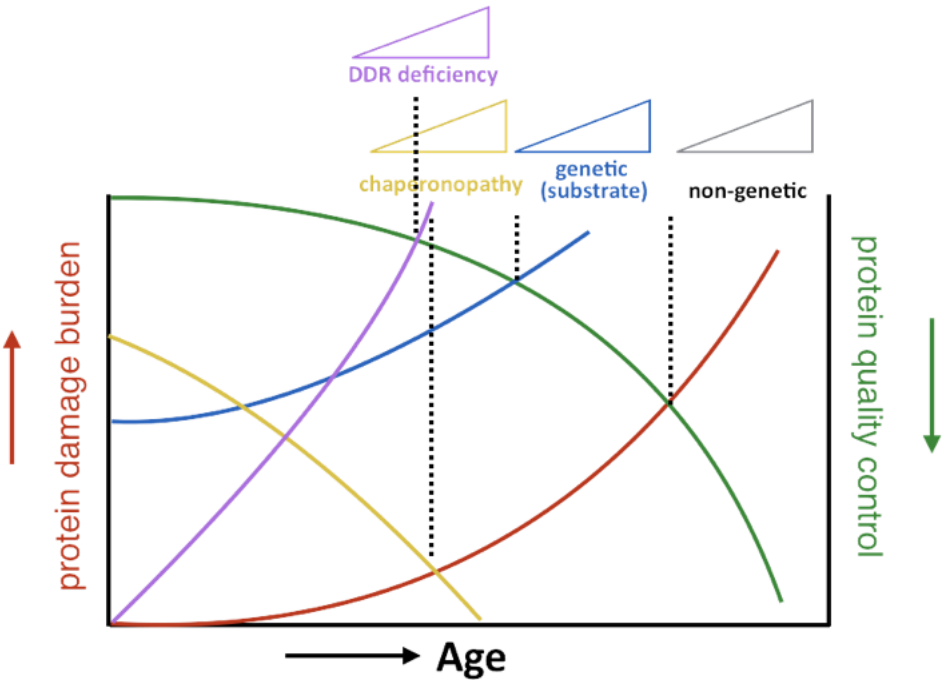

***Figure 4: Model for protein homeostasis collapse in age-related and aggregation diseases**. PQC declines with aging (green line); such decline is worsened by chaperonopathies (yellow line). The protein damage burden and risk of aggregation increases with age (red line) and is increased by factors such as pro-aggregation genetic mutations (blue line) and, likely, defects in the response to DNA damage (purple line). (Figure adapted from Kampinga and Bergink, 2016 - Heat shock proteins as potential targets for protective strategies in neurodegeneration).*

Finally, an accelerated increase in the protein damage burden may underlay the early onset of NDs in patients with mutations that lead to defects in the response to DNA damage (Figure 4 - purple line, DDR deficiency) (Madabhushi et al., 2014), although there is yet no direct evidence supporting this.

2.3. The protein aggregation process: similarities and difference between different aggregation diseases

Protein aggregates are defined here as an incorrect non-functional association of polypeptide proteins. Hereby, we want to distinguish them from large, sometimes also detergent insoluble, functional oligomeric protein complexes or from the regulated sequestration of proteins into membrane-less structures in the cell (e.g. liquid droplets or phase separations) (Banani et al., 2017).

Aggregation can be a chaotic process, as often observed under acute protein-unfolding conditions (e.g. heat shock) and when based on hydrophobic interactions. In other cases, aggregates may instead be formed during a more ordered process, as driven for example by hydrogen bonding, which usually involves the same type of protein. However, other proteins may be trapped in disordered or ordered aggregates and hereby lose their normal functionality (which is considered to be one of the mechanisms of toxicity mediated by protein aggregation) (Ross et al., 2004; Iadanza et al., 2018).

Depending on the type of aggregation-prone proteins involved in the process and on the type of intermolecular interactions that drive the incorrect protein association (e.g. hydrophobic interactions or hydrogen bonds), cellular aggregates can be distinguished in amyloid aggregates and amorphous aggregates. Mutant proteins such those containing expanded polyglutamine in PolyQ diseases, α-synuclein in PD, or amyloid precursor protein in AD can form amyloid fibrils, which are thermodynamically stable, structurally organized, highly insoluble, filamentous protein aggregates composed by repeating units of β-sheets aligned perpendicularly to the axis of the fiber and therefore with the highest level of β-sheet organization. Differently, mutant superoxide dismutase 1 (SOD-1) in Amyotrophic Lateral Sclerosis (ALS) can form amorphous aggregates, which have a low degree of β-sheet organization and are not characterized by amyloid fibrils. Importantly, the cellular conditions that triggered the aggregation process (e.g. type of proteotoxic stress, protein modifications) may determine the type of intermediates formed during the process and the aggregate morphology, finally influencing the aggregate overall cellular toxicity (Ross et al., 2004; Iadanza et al., 2018).

Below, I will focus on a selected set of NDs in which proteinaceous aggregates are found in the brain of patients: Polyglutamine (PolyQ) diseases, PD, AD and ALS. Most forms of these NDs are largely sporadic, such as AD and PD, but some have clear genetic basis (familiar forms), such as PolyQ diseases that are entirely due to a single mutation. Although age- and environmental-related factors are important contributors of the pathological process, genetic NDs are mainly driven by a specific mutation in a single gene (monogenic). The identification of the mutation in the genetic forms has provided a useful research tool to investigate the general pathological mechanisms of the NDs. Importantly, sporadic and familiar forms of the same ND, such as in the case of PD with α-synuclein aggregates (Lewy Body), show comparable histopathological features, such as type of aggregates and disease-involved brain area, making likely that both forms share a final common pathway.

Some genetic forms of NDs are recessive and the pathology is caused by the loss of normal function in the mutated protein. Notably, the most common mutations in PD have loss-of-function (LOF)

effects and occur in *Parkin (PARK2)*, *PINK1 (PARK6)*, *DJ-1* (*PARK7)* and *ATP13A2* (*PARK9*) genes, encoding for proteins involved in the UPS (e.g. Parkin is an E3 ubiquitin ligase) and mitochondrial turnover by autophagy (e.g. Parkin and PINK1) (lesage et al., 2009; Schapira et al., 2010; Martin et al., 2011; Klein and Westenberger, 2012). Yet, also this LOF subsequently leads to protein aggregation that than (further) drives the disease.

Other genetic NDs are dominantly inherited and are characterized by a mutation in a gene that causes the encoded polypeptides to be aggregation-prone and to form aggregates either intracellularly (e.g. PolyQ huntingtin in HD, or α-synuclein in PD or superoxide dismutase-1 in ALS) or extracellularly (e.g. plaques of β-amyloid in AD) with gain-of-toxic function (Imarisio et al., 2008; Kiernan et al., 2011; Guerreiro et al., 2012; Wong et al., 2017;). Although PolyQ diseases, PD, AD and ALS are all pathologically associated with protein aggregates, they are not always initiated by a protein that is intrinsically misfolded: whereas this is the case of mutant SOD-1 in ALS, mutant PolyQ proteins, for example, are not primarily misfolded, but instead need an additional processing to initiate their aggregation (Kampinga and Bergink, 2016).

Below, I will focus on genetic NDs characterized by gain-of-function mutations:

- *PolyQ diseases:*

  The PolyQ diseases are a heterologous group of trinucleotide (CAG) repeat disorders affecting proteins with entirely different function, yet leading to very similar disease phenotypes. This already indicates that LOF mechanism may not be the dominant mechanism driving these diseases. Huntington's disease (HD) is the most prevalent form of CAG repeat disorders. It is an autosomal dominant ND with the CAG repeat expansion residing in the *huntingtin* (*HTT*) gene, leading to the expression of a mutant HTT protein with an expanded PolyQ tract. HD is associated with severe motor symptoms ("chorea") and cognitive decline mainly caused by the degeneration of medium spiny neurons in the area of putamen and caudate nucleus (striatum of the basal ganglia) and various cortical regions with motor, visual and sensory functions. Intracellular aggregates of mutant PolyQ HTT are found in neurons in affected brain area of HD patients and these aggregates are considered to cause neurodegeneration via several toxic gain-of-function mechanisms (Zuccato et al., 2010; section 2.4). However, the full-length PolyQ HTT is unlikely misfolded, as it does not initiate a HSPs response (Hageman et al., 2010; Seidel et al., 2012; Seidel et al., 2016) and it does not seem a target for protein degradation (Cheichanover et al., 2015) as its steady state levels are indifferent from those of the wildtype protein (Zijlstra et al., 2010). Fragmentation of the full-length protein by cell proteases (caspases and calpains) or alternative mRNA splicing are considered key steps to initiate the formation of intracellular amyloid aggregates (Gafni et al., 2004; Haacke et al., 2007; Cowan et al., 2008).

  Spinocerebellar Ataxias (SCAs) are a set different and heterogeneous group of heritable PolyQ diseases, also characterized by the aggregation of proteins with expanded PolyQ and severe motor symptoms. This group, amongst others, includes the autosomal dominant Machado-Joseph disease (MJD or SCA3), in which intracellular amyloid aggregates of PolyQ ataxin-3 (ATXN3) are mainly found in brainstem and cerebellum (Costa et al., 2012). The role of proteolytic cleavage of PolyQ ATXN3 in SCA3 pathology is established (Berke et al., 2004; Haacke et al., 2007;

Koch et al., 2011) and ATXN3 cleavage products have been found in cellular and animal MJD models and in post-mortem brain tissues of MJD patients (Silva et al., 2018).
Dentatorubral-pallidoluysian atrophy (DRPLA) and Spinal and bulbar muscular atrophy (SBMA) are two other notable examples of PolyQ diseases. DRPLA is autosomal and dominant, associated with the mutation of the *atrophin-1* gene, presence of PolyQ aggregates, and characterized by a severe and diffuse degeneration of various regions of the brain (including cortical and sub-cortical areas), with cognitive and motor dysfunctions observed in patients (Katsuno et al., 2008). SBMA is due to the CAG repeat expansion in the *Androgen Receptor* (*AR*) gene, is inherited in an X-linked recessive manner, and is characterized by motor dysfunction and muscle weakness due to degeneration of motor neurons (Banno et al., 2012). Although SBMA is the only recessive form within the group of PolyQ diseases, it is probably the best example of pure gain-of-function protein aggregation disease. Male (XY) individuals suffering of complete androgen insensitivity do not show neurological symptoms meaning that such symptoms observed in SBMA patients are directly associated with the PolyQ expansion in the mutant AR. Interestingly, PolyQ-AR toxicity requires post-translational modifications: similarly to the normal AR, the binding with the androgen hormones induces an interdomain interaction in the mutant AR that seems associated to the propensity of the protein to aggregate and to the consequent toxicity (Zboray et al., 2015).

- *Parkinson's disease (PD)*:

PD is mainly characterized by motor symptoms due to the degeneration of dopaminergic neurons in substantia nigra pars compacta of brain basal ganglia, followed by dementia in the late phase of disease (Przedborski et al., 2017). About 5-10% cases of PD are genetic and caused by toxic gain-of-function or by the above-described loss-of-function mutations. Dominantly inherited forms of PD are those caused by mutations in or multiplication of the genes *SNCA* (*PARK1, PARK4*) (Lesage et al., 2009; Schapira et al., 2010; Martin et al., 2011; Klein et al., 2012). The mutations (e.g. A30P and A53T), as well as multiplications of *SNCA* gene, lead to aggregation of the encoded protein α-synuclein (α-syn) which is the main component of intracellular Lewy bodies and neurites, the characteristic inclusions found in PD neurons (respectively in the cell body and processes) (Wong et al., 2017). These amyloid aggregates/inclusions are also present in sporadic PD and in those caused by the LOF mutants, pointing to a central role of α-syn aggregation in PD pathogenesis. A-syn is an intrinsically disordered protein (Allison et al., 2014) that normally interacts with and binds to cellular membranes; such interaction has been suggested to be an important factor in the stability and aggregation of α-syn in PD (Zhu and Fink; 2003; Uversky and Eliezer, 2009). Abnormal covalent oxidative modifications of the protein itself may contribute to its aggregation (Schildknecht et al., 2013).

- *Alzheimer's disease (AD)*:

AD, the most common form of dementia, is mostly sporadic (although some genetic forms are also known) (Guerreiro et al., 2012) and characterized by the progressive loss of cognitive functions in patients. Common symptoms in AD are memory loss and general decline in thinking, language and learning capacity, reflecting the initial degeneration of neurons of the hippocampus region and progressive damage of other brain area. Extracellular plaques of β-amyloid and

intracellular tangles of hyper-phosphorylated tau are present in both sporadic and monogenic AD (Wolfe, 2018).

Genetic forms of AD show autosomal dominant mutations in the *amyloid precursor protein* (APP) gene. Mutant APP is normally folded and located at the plasma membrane where it requires cleavage by gamma secretase complex to generate aggregation-initiating amyloid β peptides, like amyloid β_{1-42} and amyloid β_{1-43}. Through this amyloidogenic pathways, Aβ peptides form toxic extracellular plaques of β-amyloid) (Guerreiro et al., 2012). Autosomal dominant mutations in *presenilin 1* and *2* (*PSEN1* or *PSEN2*), important components of the gamma secretase complex, are also associated with the formation of Aβ peptides (Gotz et al., 2011; Guerreiro et al., 2012). The role of β-amyloid plaques in idiopathic AD has been recently heavily challenged (Morris et al., 2014), but their elevated presence in the brain of patients with genetic AD do support the idea that can contribute in AD pathology. In fact, Aβ amyloids may exist in different strains (i.e. forming different types of aggregates), that - dependent on the patient (and maybe its PQC capacity) - may be more or less modulated or detoxified.

Sporadic and genetic forms of AD are also characterized by intracellular aggregates of tau, a protein normally involved in the stabilization of microtubules in the axons of the neurons and therefore predominantly expressed in the central and peripheral nervous systems (Wolfe, 2018). Different isoforms of tau are expressed in human brain from the alternative splicing of mRNA from the gene *MAPT.* Several mutations in the *MAPT* gene have been found in patients affected by different NDs (Ballatoreet al., 2007). Tau is subject to several post-translational modifications, and phosphorylation can reduce its ability to interact with the microtubules. Whether the phosphorylation of tau is a trigger for its aggregation still needs to be proven: aggregates of tau are always phosphorylated, but not all phosphorylated tau is aggregated; moreover there is not clear evidence that the activity of tau kinases or phosphatase is changed in AD (Iqbalet al., 2016; Wolfe, 2018). Expression in animal models of tau protein carrying disease-causing mutations, such as P301L and P301S, reproduces the typical molecular and cellular consequence observed in human disease including the formation of intracellular aggregates and neurodegeneration (Lewis et al., 2001; Allen et al., 2002).

- *Amyotrophic Lateral Sclerosis (ALS):*

ALS mainly affects the upper motor neurons (UMNs) in the motor cortex and lower motor neurons (LMNs) in the brain stem and spinal cord. LMNs communicate impulses from UMNs to muscles at the neuromuscular junctions, therefore ALS is typically characterized by a rapid and progressive loss of motor functions (i.e. control of the limbs, face muscles, jaws and tongue). Only 5-10 % of cases of ALS in humans are familiar and at least 16 genes with different dominant mutations are associated with genetic ALS (Andersen et al., 2011; Kiernan et al., 2011; Al Chalabi et al., 2012). In this Chapter, I will focus on the most frequent dominant heritable forms of monogenic ALS (about 5% of total ALS cases) associated to: 1) GGGGCC hexa-nucleotide repeat expansion in the *c9orf72* gene; 2) mutations in the *SOD1* gene (encoding for copper/zinc ion-binding superoxide dismutase 1 (SOD1)); 3) mutations in the *TDP43* gene (encoding for TAR DNA-binding protein 43 (TDP43)).

An abnormal expansion of GGGGCC hexa-nucleotide repeat in *c9orf72* gene has been recently discovered as the most common genetic autosomal dominant cause of familiar ALS. Although different hypotheses have been proposed to explain the relation between this expansion and ALS (e.g. loss of function of c9orf72 encoded protein, accumulation of toxic RNA foci), a non-exclusive mechanism is based on the evidence that repeat-associated non-ATG (RAN) translation of the *c9orf72* gene generates toxic dipeptide repeat proteins (DPRs) that are highly prone to form intracellular amyloidogenic aggregates (Freibaum et al., 2017).

About 20% of familial ALS are due to dominant mutations in the *SOD1* gene. Many mutations in *SOD1* (e.g. A4V) have been identified and nearly all can cause protein misfolding and destabilize the functional SOD1 dimer, leading to an accumulation of the monomers. Due to their exposed hydrophobic surfaces, the monomers tend to form intracellular amorphous aggregates. Aggregates of SOD1 are also found in sporadic ALS, suggesting a common pathogenic pathway with the genetic forms (Luheshi and Dobson, 2009).

Also, mutations of *TDP43* gene are associated with ALS and lead to the formation of intracellular aggregates. Notably, the aggregation of TDP43 seems one of the clearest “identifiers” of ALS and, interestingly, is also associated with the pathology of frontotemporal dementia, when degeneration occurs in the frontal and temporal lobes of the brain (Luheshi and Dobson, 2009; Andersen et al., 2011; Al-Chalabi et al., 2012).

An important aspect of the field is to unravel the mechanisms by which aggregates in NDs are toxic for the cell. In the next section, such mechanisms will be discussed with a particular attention to neurons, the brain cell type that shows a peculiar vulnerability to protein aggregation during the disease progression.

2.4. Mapping the toxicity of NDs-associated aggregates

Aggregates formed in all the above described NDs may exert their toxicity through several and potential different mechanisms, which mainly depend on the disease-associated proteins and on their cellular localization.

A detailed description of such mechanisms for each type of aggregate goes beyond the purpose of this Chapter. However, here I provide an overview of the cellular targets for toxicity of aggregates species (aggregating-prone proteins, intermediates and aggregates) associated with the above described NDs.

1) *Gene transcription and histones modifications*:
Genetic screens in cellular and *in vivo* disease models revealed that NDs, such as HD, are often associated with an altered gene transcription, although they did not always provide clear and reproducible outcomes and findings (Augood et al., 1996; Norris et al., 1996; Augood et al., 1997; Arzberger et al., 1997; Cha et al., 1998; Cha et al., 1999; Luthi-Carter et al., 2000; Chan et al., 2002; Fossale et al., 2002; Luthi-Carter et al., 2002a; Luthi-Carter et al., 2002b; Sipione et al., 2002; Hodges et al.,2006). A pleiotropic alteration in transcription could likely be a downstream consequence of protein aggregation in other cellular sites. Alternatively, but not mutually exclusive, aggregates, such as observed with HTT aggregates, are known to sequester specific transcription factors (TFs), which might finally contribute to cellular dysfunction and degeneration. The functional consequences of aggregate toxicity strongly depend on the type of TFs trapped (Boutell et al., 1999; Shimohata et al., 2000; Steffan et al., 2000; Holbert et al., 2001; Nucifora et al., 2001; Dunah et al., 2002; Zhai et al., 2005; Zuccato et al., 2007; Cui et al., 2006). Aggregates also trap chromatin regulators (i.e. histone-modification enzymes), hereby changing the epi-genetic landscape and leading to a more global change in gene expression profiles and hence neuronal functionality (Steffan et al., 2001; Sadri-Vakili et al., 2007).

2) *Nucleocytoplasmic transport*:
The nuclear pore complex (NPC) is a protein complex that controls the fundamental nucleocytoplasmic transport of RNA molecules and proteins. Recent data have revealed that dysfunction in NPC transport could be a very early effect in many different NDs aggregation diseases (Shur et al., 2001; Lee et al., 2006; Sheffield et al., 2006; Jovicic et al., 2015; Zhang et al., 2015; Freibaum et al., 2015; Zhang et al., 2016; Grima et al., 2017;). Such early event will interfere and even disrupt the normal function of NPC and may actually have a high self-propagating nature as a disrupted nucleocytoplasmic transport will impede on nearly all metabolic and even many catabolic processes in the cell.

3) *RNA metabolism*:
RNA binding proteins (RBPs) are responsible for the mRNA maturation in the nucleus (in processes such as splicing, capping and nuclear export) and its translation in the cytoplasm. RBPs and transcripts transiently form different types of granules in nuclei and cytoplasm, which are essential for RNA metabolism. For example, processing bodies (P-bodies) are involved in mRNA silencing and degradation (Maziuk et al., 2017), whereas stress granules (SGs) are formed during cell stress (e.g.

heat shock, oxidative stress and ER stress) to silence non-essential transcripts and promote the translation of stress-response proteins such as HSPs (Maziuk et al., 2017). Transcripts are incorporated in SGs together with RBPs and other proteins that enable their interaction with the cytoskeleton and other organelles; importantly, RNA granules in neurons are also involved in mRNA transport along the axons for the final translation at the synapses (Maziuk et al., 2017). A typical RBP contains a RNA binding domain, nuclear import-export sequences and "low complexity" domains (LCDs), which mediates the formation of RNA granules (King et al., 2012). RNA granules are highly dynamic entities that readily disassociate to release the transcripts. A number of disease related proteins (in particular those causing ALS, such as TDP43) are normal constituents of these RNA granules. In disease, the presence of these mutated proteins reduces SG dynamics, likely because they now rapidly transform from a liquid to crystal phase (as highly stable amyloids). Such mechanism impedes on the physiological function of these granules and can be toxic to the cells (Mori et al., 2013; Liu-Yesucevitz et al., 2014; Kwon et al., 2014; Lee et al., 2016; Lin et al., 2016; Conicella et al., 2016).

4) *HSPs*:

As previously explained, HSPs play a central role in protein homeostasis and are the first-line of defense against protein aggregation. HSPs activity or inducibility declines with aging which may enhance the susceptibility to protein aggregation (Higuchi-Sanabria et al., 2018). Indeed, mutations in chaperones and hence impairment of protein quality control has been associated with neuro- and muscular degeneration associated with aggregation (Macario and Conway de Macario, 2002; Macario et al., 2005; Kakkar et al., 2016). Different HSPs are frequently found/recruited to NDs-associated aggregates. For example, HSP70 and HSP40 members are found in PolyQ inclusions (Wyttenbach et al., 2000; Suhr et al., 2001; Waelter et al., 2001). This might be due to the fact that HSPs recognize and interact with these aggregate species. However and inversely, aggregate species may sequester the HSPs in or at the aggregate (trapping) which may cause an impairment in the activity of HSPs further accelerating protein aggregation of the disease-relevant protein.

5) *Ubiquitin-Proteasome System (UPS):*

Similar to HSPs, the pathology of many NDs is associated with the reduced activity of the UPS capacity, which may occur during aging and result in a reduced capacity to degrade unfolded and misfolded proteins, perpetuating the formation of toxic aggregates. Several mutations in UPS components affect the ubiquitin-dependent processes and are linked to neurodegenerative processes and presence of protein aggregates: two examples are the ubiquitin-ligase Parkin, mutations in which cause an autosomal recessive form of PD (Kitada et al., 1998), and the de-ubiquitylating enzyme ATXN3, mutations of which are responsible of SCA3 (Evers et al., 2014). In these NDs, whereas the proteasome remains operative, the ubiquitination of the substrates is largely impaired, leading to reduce protein degradation and increased risk of protein aggregation. However, UPS can also be considered as a potential target of aggregate toxicity because aggregates species are capable to inhibit the activities of UPS components, therefore sustaining the pathological mechanism in a positive feedback loop (Ciechanover et al., 2015; Dantuma et al., 2014). There is evidence that aggregates (such as HTT and α-syn) may directly impede the proteasomal activity through direct interaction with its subunits (Stefanis et al., 2001; Snyder et al., 2003; Lindersson et al., 2004; Chen et al., 2006a; Diaz-Hernandez et al., 2006). Other studies suggest that

PolyQ proteins - which are poor substrates for the UPS - get stuck in proteasomes (clogging) (Homberg et al., 2004). However, more recent data rather suggest that such proteasomal impairment may be a late event in the diseases (Seidel et al., 2012; Seidel et al., 2016) and the result of an overall and complex imbalance of protein homeostasis, resulting in a proteasomal overload (Dantuma et al., 2014).

6) *Autophagy*:
Autophagy is an important pathway of removal of mis- or unfolded proteins, aggregates and damaged organelles such as mitochondria (via mitophagy). Notably, neurons retrogradely transport the cargo (e.g. aggregates and senescent mitochondria) from their axon and neuronal termini to their soma through a long and complex process, the efficiency of which is affected by aging and aggregates in NDs (Ciechanover et al., 2015; see also point 11). As in the case of HSPs and UPS, reduced autophagy is observed in aging and may contribute to NDs pathogenesis. Moreover, genetic impairment of autophagy has been shown to lead to neurodegeneration (Hara et al., 2006). Autophagy is often found to be impaired in NDs, as revealed by an abnormal accumulation of autophagosomes or reduced lysosomal activity in degenerating cells (Cuervo et al., 2004; Nixon et al., 2005; Morimoto et al., 2007; Boland et al., 2008; Martinez-Vicente et al., 2010; Wong et al., 2010; Nixon et al., 2011; Lee et al., 2012;). Disease-associated aggregates have been shown to impair autophagy, through the direct interactions of the toxic species with the autophagic components, such as α-syn and HTT that have a high affinity with the autophagic protein complex LAMP-2A on the lysosomal membrane (Cuervo et al., 2004; Malkus et al., 2012; Qi et al., 2012). As result, the normal autophagic cycle may be blocked as suggested by the accumulation of autophagosomes and lysosomal impairment.

7) *Ca^{2+}homeostasis*:
In neurons, Ca^{2+} regulates the activity of several responsive proteins like Ca^{2+}-dependent enzymes (e.g. calpains and adenylate cyclases) and proteins involved in cellular signaling (e.g. calmodulin, kinases and phosphatases), gene transcription (e.g. calcineurin and cAMP response element binding protein (CREBP)), cytoskeleton dynamics (e.g. dynein) and synaptic functionality (e.g. synaptotagmins). Moreover, Ca^{2+} has a key role in neurotransmission and in the short- and long-term synaptic plasticity. Therefore, neurons control the intracellular levels of Ca^{2+} by carefully regulating the activity of different Ca^{2+}channels on the plasma membrane (e.g. NMDA and AMPA receptors, voltage-gated Ca^{2+} (VGCCs) and TRP channels, plasma membrane Ca^{2+} pump (PMCA) and Na^{+}/Ca^{2+} exchanger (NCE)) and on the membranes of Ca^{2+}store-organelles like the ER and mitochondria (e.g. InsP3 and Ryan receptors, the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and the mitochondrial calcium uniporter (MCU)) (Bezprozvanny et al., 2009). A disturbance in Ca^{2+} homeostasis is observed in several NDs including HD, PD, AD and ALS (Bezprozvanny et al., 2009). Aggregate species associated with NDs, such as in the case of PolyQ-HTT and ATXN3 in HD and SCA3 respectively, disturbs the Ca^{2+} homeostasis by interfering with the normal activity of some of the channels located at the plasma and organelle membrane (Zeron et al., 2002; Tang et al., 2003; Swayne et al., 2005; Tang et al., 2005; Shehadeh et al., 2006; Fan et al., 2007; Kaltenbach et al., 2007; Chen et al., 2008; Zhang et al., 2008) or otherwise by forming transmembrane Ca^{2+} permeable pores in the bilayer (see point 13) (Buttrefield et al., 2010). This primarily determines a dysregulation of the levels of Ca^{2+} in the different cell compartments and consequently of the Ca^{2+}-dependent

pathways at the plasma membrane, ER (see point 8) (Hetz and Saxena, 2017; Remondelli and Renna, 2017), Golgi (see point 9) (Machamer et al., 2015), mitochondria (see point 10) (Lin and Beal, 2006; Federico et al., 2012) and synapses (see point 12). Slight changes in Ca^{2+} levels also further aggravates aggregation of proteins by modulating the activity of proteases like calpains and caspases that trigger the abnormal cutting of proteins in peptides with a high propensity to aggregate (e.g., huntingtin in HD) (Gafni et al., 2004; Cowan et al., 2008; Haacke et al., 2007).

8) *ER:*

The ER is a site of folding for at least one-third of the proteome. In NDs, the accumulation of protein aggregate species has been shown to cause chronic UPR^{ER} activation, impairing the normal functionality of the organelle in proteins maturation, trafficking and degradation. Like for several of the before mentioned effect of aggregation, ER impairment due to aggregate species may cause a forward vicious cycle of decline in protein homeostasis (Hetz and Saxena, 2017; Remondelli and Renna, 2017) .Several mechanisms by which aggregate species can interfere with ER homeostasis have been identified. For example, aggregates disrupts the normal functionality of Ca^{2+} channels on the ER membrane (e.g. InsP3R) and consequently the overall Ca^{2+}-based regulation of ER and cytosolic proteins (see also point 7) (Tang et al., 2003; Higo et al., 2003; Belal et al., 2012; Selvaraj et al., 2012). Aggregates also have been shown to interact and interfere with the functions of ERAD components, therefore interfering with the normal degradation of proteins (Nishito et al., 2008; Yang et al., 2010; Abisambra et al., 2013). The ER-Golgi trafficking is altered by aggregate species through their interference with the normal functions of proteins involved in vesicles tethering, docking and fusion (e.g. Rab GTPases) (Cooper et al., 2006; Gitler et al., 2008), leading to a further toxic accumulation of proteins in the organelle. Finally, aggregate species also interfere with the UPR^{ER} pathway via its signal transducers (e.g. ATF6 in HD) (Fernadez-Fernandez et al., 2011; Naranjo et al., 2016).

9) *Golgi apparatus (GA) and vesicular trafficking*:

Through a finely regulated vesicular trafficking, proteins are transported from the ER to the GA where they are processed via post-translational modifications (e.g. glycosylation, proteolytic cleavage) and sorted to different compartments and membranes (including the plasma membrane, the extracellular space and the endo-lysosomal system). Notably, Golgi outposts are also present in neuronal axons and dendrites and may have an important role for the protein trafficking in these cellular sites. The GA structure, consisting of stacks of parallel cisternae, is primarily maintained by the microtubules of cytoskeleton, GRASPs and golgins and can be reversibly disassembled (fragmentation) during physiological cellular process (e.g. mitosis) (Machamer et al., 2015; Gonatas et al., 2006). Interestingly, GA fragmentation is also often observed before the degeneration of neurons in NDs and in concomitance with protein aggregation, although the link between the two has not been elucidated (Gonatas et al., 1992; Stieber et al., 1996; Mourelatos et al., 1996; Gosavi et al., 2002; Huse et al., 2002; Stieber et al., 2004; Gonatas et al., 2006; Gujita et al., 2008; Tong et al., 2012; Joshi et al., 2014; Baloyannis et al., 2014; Van Dis et al., 2014). Such irreversible fragmentation of the GA negatively impacts on the trafficking and processing of many essential proteins and membranes) and therefore can significantly contribute to NDs aetiology (Gonatas et al., 2006; Machamer et al., 2015). NDs-associated aggregate species perturb the homeostasis of many GA-specific proteins involved in vesicle trafficking or GA structure , leading to an abnormal

accumulation of proteins in GA and its final fragmentation (Stieber et al.,2004; Sundaramoorthy et al., 2013; Atkin et al., 2014; Soo et al., 2015; Sundaramoorthy et al., 2015).

10) *Mitochondria:*
Mitochondria provide energy through the production of ATP and are involved in metabolism and Ca^{2+} homeostasis (see also point 7). Importantly, they contain several antioxidant molecules and enzymes (e.g. glutathione, coenzyme Q10, catalase and glutathione peroxidase), which inhibit the toxicity of oxidant species produced during mitochondrial respiration (Lin et al., 2006b; Federico et al., 2012). Mitochondrial dysfunctions have been observed in most NDs and acknowledged to be able to trigger cell degeneration and such as may contribute to the onset of the disease (Jenkins et al., 1993; Gu et al., 1996; Kong et al., 1998; Nunomura et al., 2001; Pratico et al., 2001; Mattiazzi et al., 2002; Damiano et al., 2006; Shapira et al., 2008). Importantly, mitochondrial dysfunctions can cause the accumulation of reactive oxygen species (ROS), causing oxidative stress, which could perpetuate and augment aggregate initiated damage by causing damage to the nuclear and mitochondrial DNA, to lipid membranes (e.g. through peroxidation, causing membrane breakage), to membrane proteins (causing leakage or impair mitochondrial import), and to soluble proteins (e.g. through fragmentation and oxidation, promoting their aggregation) (Lin and Beal 2006; Federico et al., 2012). NDs-associated aggregates species are known to alter the normal functions of the organelle (e.g. by interaction with its membranes, enzymes and membrane proteins) and, in some cases, interfere with its dynamics (e.g. fusion, fission, degradation via mitophagy and organelle movements) (Casley et al., 2002; Mattiazzi et al., 2002; Anandatheerthavarada et al., 2003; Choo et al., 2004; Liu et al., 2004; Lustbader et al., 2004; Park et al., 2004; Pasinelli et al., 2004; Song et al., 2004; Crouch et al., 2005; Vijayvergiya et al., 2005; Manczak et al., 2006; Martin et al., 2006; Chinta et al., 2008; Orr et al., 2008; Song et al., 2011; Luth et al., 2014; Di Maio et al., 2016).

11) *Axonal transport and cytoskeleton*:
Neurons have a unique cellular morphology and rely on axonal transport for their maintenance and functions: several types of cargoes are transported along the microtubules of the axon cytoskeleton, with the direct participation of microtubule-associated proteins (e.g. Tau) and by several motor proteins like kinesins (for the anterograde “soma-synapse” transport) and dynein complexes (for the retrograde “synapse-soma” transport). Proteins, Golgi-derived vesicles, neurosecretory granules (containing molecules and proteins like neurotransmitters and neurotrophic factors), mRNA (to be translated at the synaptic terminal) and mitochondria are transported anterogradely; senescent mitochondria, autophagosomes (destined to degradation via autophagy in the soma, see also point 6) and endosomal recycling vesicles are instead transported retrogradely (Millecamps et al., 2013). Early events, observed in almost all NDs, are the defective axonal transport and accumulation of the cargoes in the soma, in the axon and in the synaptic terminal, which have been often linked to the presence of protein aggregates that physically may obstruct all these transport processes (Wagner et al., 1996; Patrick et al., 1999; Nguyen et al., 2001; Kamal et al., 2001; Saha et al., 2004; Gauthier et al., 2004; Ackerley et al., 2004; Dompierre et al., 2007; Morfini et al., 2009; Bosco et al., 2010). Interestingly, the morphological features of neurons in several aggregate-associated NDs - the so-called ballooned neurons - resembles the swollen neurons produced by neurotoxins that impairs the axonal transport.

12) *Synapses*:
Whilst several of the above-mentioned toxic effects of aggregates may indirectly affect the synaptic functionality (e.g. altered Ca^{2+} homeostasis, transcription dysregulation, disruption of axonal transport, damage of the phospholipid bilayer), aggregates can also exert a direct toxicity on synapsis. For example, α-syn aggregates can interfere with the synaptic vesicle maturation and trafficking, with the normal function of synaptic proteins (e.g. SNAREs) and with the regulation of neurotransmitters release and re-uptake (Masliah et al., 2000; Chung et al., 2009; Garcia-Reitbo et al., 2010; Nemani et al., 2010; Lundblad et al., 2012; Scott et al., 2012; Choi et al., 2013; Janezic et al., 2013; Wang et al., 2014).

13) *Membranes*:
Intracellular and extracellular aggregate species can induce the disruption of the cellular membranes through their direct interactions with the phospholipid bilayer (Arispe et al., 1993; Jang et al., 2010; Reynolds et al., 2011; Bäuerlein et al., 2017). The main cytotoxic effects of such interactions are the modifications of chemical-physical properties (e.g. rigidity and conductance) and permeabilization of the membrane. Different mechanisms have been suggested to explain how aggregate species can destroy the bilayer: A) deformations and possible rupture of the membrane due to the interaction and physical impinge of the growing aggregate on the bilayer; B) detergent-like micellization, during which the monomers of toxic protein are adsorbed by the bilayer and, subsequently, the growing aggregate causes lipid extraction and membrane thinning; C) formation of transmembrane pores during which aggregates species are incorporated in the bilayer and form a channel which is permeable to molecules and ions (see also point 7). These mechanisms might involve both the plasma membrane and those of organelles, causing impairments in their organization and dynamics (Butterfield et al., 2010).

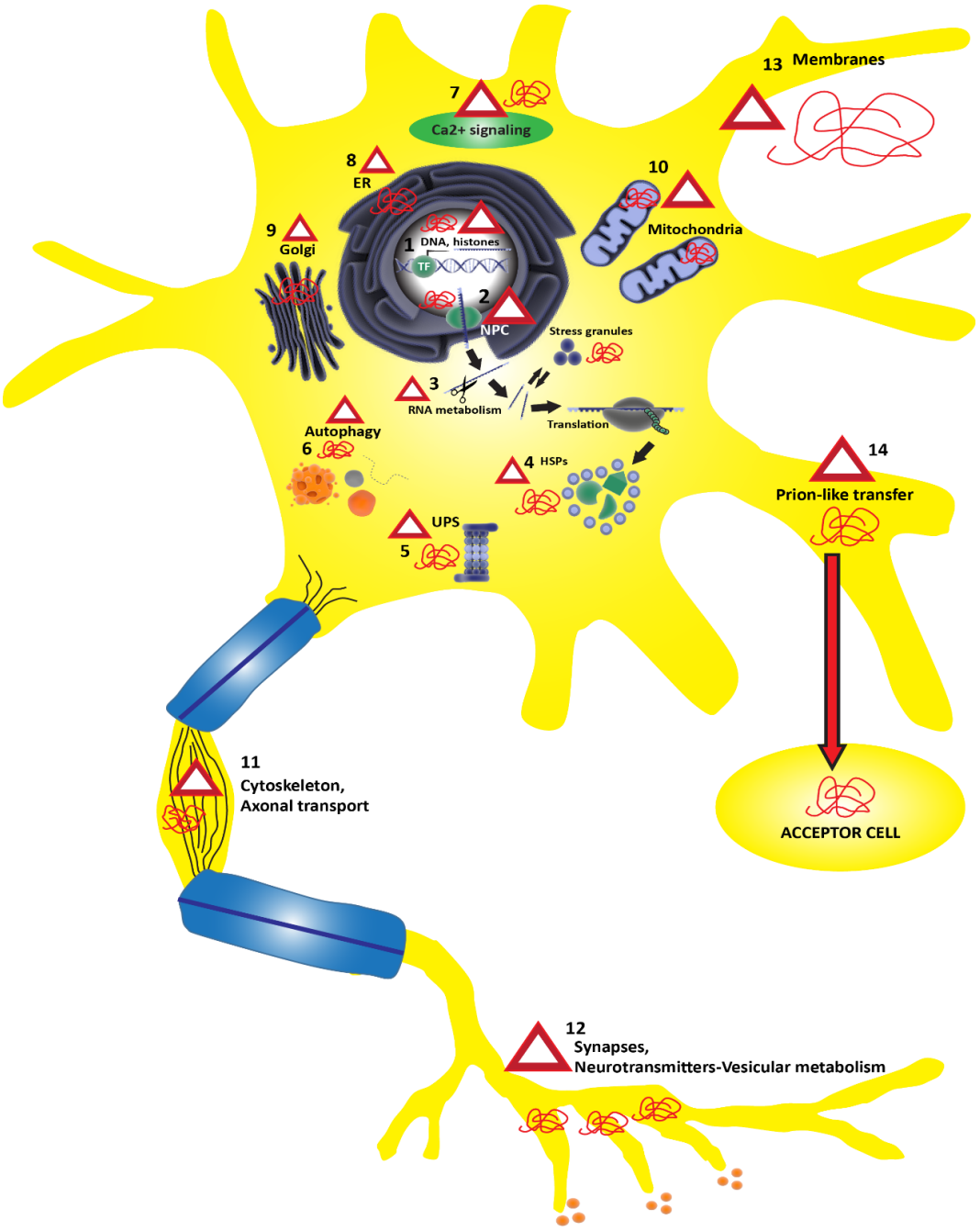

Figure 5: Mapping the toxicity of NDs-associated aggregates. *The main sites/pathways of damage in neurons mediated by ND-associated aggregates are: 1) Gene transcription (including transcription factors) and histones; 2) Nucleocytoplasmic transport; 3) RNA metabolism; 4) HSPs; 5) Ubiquitin-Proteasome System (UPS); 6) Autophagy; 7) Ca^{2+} homeostasis; 8) Endoplasmic reticulum (ER); 9) Golgi apparatus (GA) and vesicular trafficking; 10) Mitochondria; 11) Axonal transport and cytoskeleton; 12) Synapses; 13) Membranes. Aggregate-mediated damage at these sites contributes to neuronal degeneration. The same toxic protein can form aggregates at different cellular sites and can affect multiple pathways. Dysfunctionalities in some of these pathways contributes to an alteration of protein homeostasis (e.g. HSPs, UPS, autophagy and Ca^{2+} homeostasis), therefore perpetuating the formation of toxic aggregates. NDs associated aggregates have also prion-like properties (14) (see section 2.6).*

Given such a multitude of cellular damages that may result from aggregates in NDs (summarized in Figure 5), it is unlikely that strategies that focus to correct either one of these targets individually

may produce long-lasting therapeutic effects in patients. For this reason, many research groups, including ours, have focused on the development of anti-aggregation therapeutic strategies that aim to prevent or delay the formation of these toxic species before they might initiate this cascade of these different toxic (and often self-perpetuating) processes.

2.5. Neuronal vulnerability in aggregate-related diseases

Neurons show a peculiar vulnerability to protein aggregation (Saxena et al., 2011). In some genetic NDs, although the disease-causing mutant protein is ubiquitously expressed in all cells of the body (such as in the case of PolyQ HTT in HD; Zuccato et al., 2010), neurons in particular show early onset pathology (neuronal vulnerability). Moreover, such vulnerability is often initially restricted to a specific sub-population of neurons (selective neuronal sensitivity). Even more peculiar, different PolyQ diseases, such as HD and SCA3, are both caused by a mutant protein with an expanded PolyQ stretch (which drives protein aggregation and disease), but whereas PolyQ HTT in HD primarily affects striatal neurons (Zuccato et al., 2010), PolyQ ATXN3 in SCA3 primarily affects cerebellar Purkinje cells (Evers et al., 2014).

The cause for these selective neuronal sensitivities is still not clear. Several research groups have characterized the transcriptome and proteome of different subgroups of neurons, aiming to identify specific candidates that could be linked to hypersensitivity of specific neurons to the pathogenic process. Differences have been found, but no clear marker explaining the vulnerability of a specific subset of neurons to a specific mutant protein has been identified so far (Mattson et al., 2006).

Nevertheless, the studies regarding the biology of neurons, the cellular PQC (see section 2.1) and the mechanisms by which aggregates can be toxic for cells (and particularly for neurons, see section 2.4) suggest that some of the following factors may contribute to the higher vulnerability of neurons to these diseases (independently from which neurons and which neuronal areas):

- *The distinct morphology:* neurons (and particularly the vulnerable subgroups) have very long axons that may connect many different CNS regions with each other (e.g. striatum of the basal ganglia and cortical regions in HD) or the CNS with the periphery (e.g. motor neurons in ALS) (Mattson et al., 2004; Zuccato et al., 2010; Kiernan et al., 2011). Such long axons require a high trophic support and imply a larger cell surface area exposed to toxic environmental conditions. The peculiar presence of an axon in the neuron also implies the presence of synaptic terminals which depend on an efficient transport of molecules and organelles from the very distant soma across a small space that easily may be affected by aggregates.

- *The metabolism with high energy requirements*: neurons are extremely energy-demanding cells (Belanger et al., 2011) and the high oxidative metabolism might contribute to a greater formation of ROS. For these reasons, the interlinked mechanisms of protein aggregation due to ROS-mediated modifications and aggregate toxicity on mitochondria (that increases levels of ROS) might be particularly exacerbated in neurons.

- *Post mitotic:* neurons are post-mitotic cells with no or extremely limited capacity of division and cell replacement (although few neuronal stem cells with such capacities are present in human CNS). Therefore, differently from other cells, neurons lack the protective strategy of asymmetric partitioning of aggregates during cell division and the possibility to replace damaged neurons.

- *The responsiveness to neurotransmitters*: the plasma membranes of neurons are rich of receptors for different neurotransmitters and, notably, the most vulnerable subgroups are particularly responsive to excitatory neurotransmitters such as glutamate and dopamine (e.g. striatal medium spiny neurons in the area of putamen and caudate nucleus in HD) (Mattson et al., 2006; Saxena et al., 2011). During excitotoxicity, post-synaptic receptors are over-activated by abnormal higher levels of glutamate, leading to increased influxes of Ca^{2+} that may trigger a-specific activation of Ca^{2+}-dependent proteases to fragment disease-related proteins or otherwise trigger their unfolding (section 2.4, point 7).

- *The activity of the neuronal PQC*: the capacities of HSPs and protein degradation systems (UPS) might be intrinsically lower in neurons compared to other non-neuronal cells (section 3.4).

All together, these data indicate that the intrinsic cellular and molecular characteristics of neurons make them particularly vulnerable to protein aggregation in NDs.

2.6. Prion-like properties of NDs-Associated aggregates

In the previous section, we concentrated on how aggregates are formed *in situ,* in a compartment or organelle where they directly exert their toxicity ("cell-autonomous" effects). However, recent evidence *in vitro* and *in vivo* showed that NDs-associated aggregates may also have an *ex situ* origin (Brundin et al., 2010; Costanzo et al., 2013a).

Prion diseases (PrDs) are characterized by severe neurodegeneration and neuroinflammation and are caused by proteinaceous agents named "prions", which consist of pathological aggregates (PrP^{Sc}) of the prion protein PrPc, a plasma membrane protein. The aggregation nucleus can entrap wildtype PrPc monomers, often referred to as conversion, and PrP^{Sc} oligomers grow. Such oligomers can be fragmented, thus forming new nuclei, each of which can restart the nucleation and fragmentation cycle. The minimal self-replicating unit of aggregates is defined *propagon* and during the aggregate amplification process, normal proteins can be sequestered through co-aggregation. Prions show a great resistance to degradation by cellular proteases, heat and other denaturing agents. Human prion diseases typically affect the brain and neurons show a peculiar vulnerability. The most striking characteristic of prion diseases is the transmissibility of the proteinaceous agent between hosts: in the transmissible spongiform encephalopathies (TSEs), for example, transmission of the disease occurs when an individual is "infected" by prions mainly through ingestion of tissues or blood transfusion from another affected individual (Scheckel and Aguzzi, 2018). Prions are capable to reach the brain via the gut-brain axis where they can penetrate in neurons to start the aggregation cycle, co-sequestering the endogenous protein. Notably, experiments in animal models

confirmed that the normal protein is initially required for the prion disease to occur, suggesting that the prion must interact with the normal endogenous protein to initiate the vicious aggregation cycle.

As reviewed by Brundin, Melki and Kopito in 2010, an increasing amount of evidences suggests that also aggregates associated with the above-mentioned NDs, such as PD, HD and AD, might spread in a 'prion-like manner' and that disease progression is associated with the intercellular transfer of pathogenic proteins (Brundin et al., 2010).

This had already for long been suggested by the anatomical patterns of degeneration that are specific for each disease (Braak et al.,2003). Post-mortem analysis of brains from NDs patients showed that, whereas the disease starts in specific brain sites, protein aggregation and neuronal degeneration progresses over time to other brain regions, displaying a stereotypical anatomical pattern that is typical for each NDs and that follows specific networks of synaptic circuits as described below.

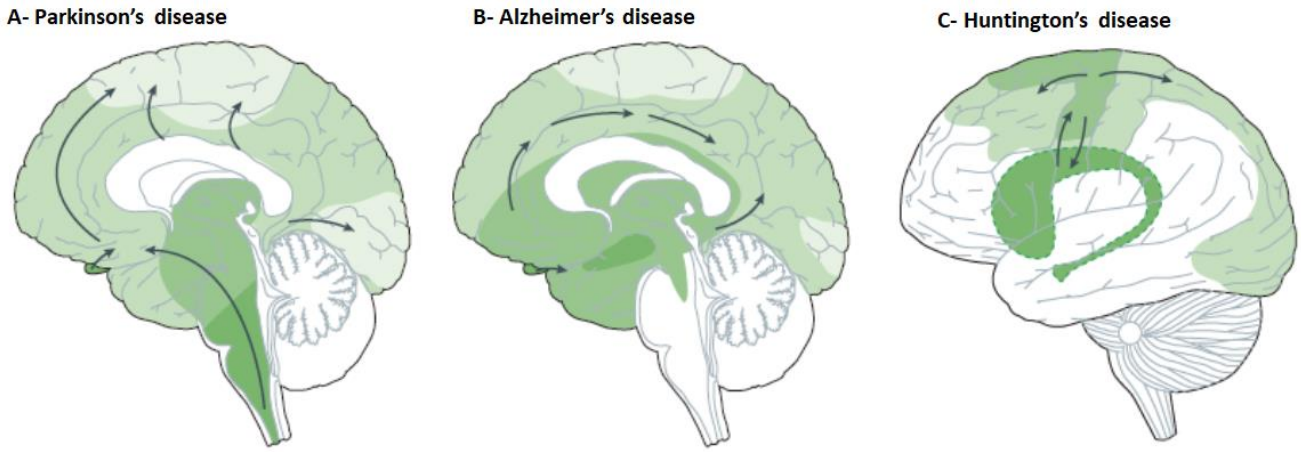

***Figure 6**: how neuropathological changes in Parkinson's, Alzheimer's and Huntington's diseases spread spatiotemporally during disease progression. The earlier the neuropathology develops in a given brain region, the darker the shading in the figure. (Image retrieved from Brundin et al., 2010 - Prion-like transmission of protein aggregates in neurodegenerative diseases)*

In HD, the area of putamen and caudate nucleus have been suggested to be the first to degenerate and show PolyQ HTT aggregates, although other studies indicate that motor and sensory cortical regions are also early affected by neurodegeneration (Zuccato et al., 2010). In MJD, PolyQ ATXN3 aggregation and neurodegeneration initially involve brainstem nuclei and cerebellum. In a later phase, these can be widespread and variable throughout many other region of CNS (Paulson et al., 2017).

In early-phase PD, Lewy bodies and neurites of α-synuclein appear and neurodegeneration occurs in the olfactory bulb, in the anterior olfactory nucleus and in the dorsal motor nucleus of the vagus nerve in the medulla oblongata (Brettschneider et al., 2015). Starting from the degeneration in these areas, other regions of the CNS (such as pons and midbrain) are affected in a later phase. Notably, the distinctive motor symptoms of PD are mainly associated with the degeneration of dopaminergic

neurons in the midbrain during the late phase. Interestingly, in early PD, α-synuclein aggregates and pathology have even been detected in peripheral neurons of the enteric nervous system, which are connected with the CNS through the brain-gut axis.

In AD, intracellular tangles of tau are first found in the hippocampus, in other regions of the temporal lobe and in the brainstem. Later, they also appear in the cortical regions (insular cortex and neocortex). The appearance of extracellular plaques of β-amyloid follow instead the opposite direction, earlier in the cortex and later in the brainstem. Although tau and β-amyloid are both connected with AD pathology and neurodegeneration, the reasons of this opposite pattern of spreading in the brain still need to be elucidate (Brettschneider et al., 2015).

Also in ALS, specific patterns of aggregation and degeneration of neurons have been recognized. However, it is still not clear whether ALS pathology starts in motor neurons of the cortex and brain stem/spinal cord (substantiating the "dying-forward" hypothesis) or in muscle cells (for the "dying-back" hypothesis) (Kiernan et al., 2011).

Different reasons for the domino-like degeneration of neuronal cells in such diseases have been proposed:

- Connected neurons normally inter-exchange molecules (including neurotransmitters), growth factors and anti-apoptotic signals for functionality, viability and support. Synaptic disruption leads to failure of this trophic support and "spreading" of degeneration along neuronal circuits. Loss of neurons may also dysregulate the pre- and post-synaptic transmission, triggering the degeneration of neighbor neurons (Mattson et al., 2006).
- Shared vulnerability of neuronal networks might be due to intrinsic cell autonomous factors (e.g. gene expression, neurotransmitters sensitivity, position in the circuit), that modulate a specific susceptibility (or resistance in no affected area) against the disease, although no clear markers have been identified so far (section 2.5.).
- Lastly and as discussed further below, protein aggregates associated with different NDs may spread between cells in a prion-like manner suggesting an intriguing explanation for this circuits-specific disease progression (this section).

Beside the observation of such anatomical patterns of neurodegeneration (Figure 6), experimental evidences for the "prion-like theory" were also initially provided by the injection of Aβ or tau or α-syn aggregates, obtained from the brain of AD or PD patients, into the brain of mice. This later caused the aggregation of the endogenous wild-type mouse protein in brain regions also really distant from the injection site (i.e. from one hemisphere to the contralateral one) (Claguavera et al., 2009; Eisele et al., 2010; Luk et al., 2012, Mougenot et al., 2012). Further, brain autopsies from PD patients, who received transplant of healthy embryonic neurons, showed that a subset of these engrafted cells displayed α-syn aggregates (Kordower et al., 2008a; Kordower et al., 2008 b; Li et al., 2008; Li et al., 2010). Similarly, Cicchetti and colleagues reported that neurons transplanted in HD patients showed disease-like degeneration (Cicchetti et al., 2009). Although they initially did not report HTT aggregates in these transplanted and degenerating neurons, they later re-examined the same cases using new techniques and they showed that the grafts do contain mutant HTT (Cicchetti

et al., 2014), providing evidence that also PolyQ HTT in HD shows prion-like properties similarly to α-syn in PD.

In vitro data have then shown that NDs-associated aggregates can be released from donor cells and enter in acceptor cells in which they are capable to initiate aggregation. "Initial" donors might be those neurons which are more vulnerable to protein aggregation (i.e. neurons in putamen and caudate nucleus in HD; neurons in hippocampus in AD), whereas acceptors might be neighbor cells (i.e. interneurons and glial cells) or neurons with synaptic contacts with the initial donor cells. In these *in vitro* experiments, two populations of cells are co-cultured using a specific culture system (i.e. "classic" co-culturing-, trans-well culturing- or conditioned media- experiments): one population (the donor cells) expresses the toxic protein usually tagged with a fluorescent protein (i.e. GFP) whereas the other co-cultured population (the acceptor cells) expresses the normal protein tagged with a different fluorescent protein (i.e. RFP). Transcellular spreading of the GFP-tagged aggregate species is evidenced by their appearance in the RFP fluorescent cells. Moreover, the presence of yellow fluorescent puncta suggests seeding and co-aggregation of the normal protein in the acceptor cells and ultimately the prionoids property of the ND-associate protein under study. In other experiments, prionoids are added to the cell medium, and their entering and seeding in the acceptor cells are verified after a certain time of incubation (Costanzo et al., 2013a).

Such studies have been first conducted using non-neuronal cells, like the human cell line HEK293 to investigate the prionoids properties of a certain aggregate; later the same studies have been repeated in neuronal cell lines from humans and rodents, finally demonstrating that α-syn, tau, SOD-1, TDP-43, DPRs (from *c9orf72* mutated gene), HTT and ATXN can enter in the cells in a prion-like manner (see table 1).

Five basic requirements have been identified to fulfill the criteria of a prion-like mechanism (Brundin et al., 2010; Costanzo et al., 2013a):

1. The protein aggregate must be capable of elongating by the recruitment of soluble polypeptide chains and of fragmenting to generate additional elongation sites and amplify aggregation.
2. The aggregates must be released from the donor cells. Several mechanisms have been proposed and are currently under investigation. These include: a) passive release of aggregates by membrane breakage during or after the donor cell degeneration; b) active release of aggregates via exocytosis, or as cargo in exosomes or as cellular material in exophers (Melentijevic et al., 2017).
3. The aggregates must be able to enter in the acceptor cells. Several mechanisms for the entering of NDs-aggregates have been proposed such as: a) passive uptake (i.e. via the disruption of the plasma membrane); b) active mechanisms of endocytosis and phagocytosis; c) movement of the aggregates via cell-to-cell membrane nanotubes. Importantly the internalized aggregate must be able to escape the endosomal system in order to interact with the endogenous protein and cause seeding and aggregation
4. Acceptor cells, which are receiving the aggregates, must express the non-aggregated form for the "infectious" aggregate species to initiate the aggregate amplification process (aggregate *seeding*). Notably, the differential expression of the protein in different cells might partially

explain the specific vulnerability/resistance against aggregation: cells with lower expression of the protein might be more resistant to the seeding.

5. The aggregates must be resistant to degradation, especially during their cell-to-cell movement in the extracellular space (i.e. in the synaptic cleft).

Table 1: Pathogenic proteins in NDs are prionoids. *In vitro and in vivo studies showing that NDs-associated aggregates in HD (HTT), AD (A-Beta, tau), PD (α-syn) and ALS (DPR proteins, TDP43 and SOD1) have prion-like characteristics.*

Neurodeg. disease	***In vitro, in vivo* studies and findings in patients**
Alzheimer's (Aβ)	**IN VITRO (cellular models):** • **Nath S, et al. 2012 -** Spreading of neurodegenerative pathology via neuron-to-neuron transmission of β-amyloid. • **Sardar et al., 2018** - Alzheimer's disease pathology propagation by exosomes containing toxic amyloid-beta oligomers. **IN VIVO:** • **Meyer-Luehmann et al., 2006** - Exogenous induction of cerebral β-amyloidogenesis is governed by agent and host. • **Eisele et al., 2010** - Peripherally applied Aβ-containing inoculates induce cerebral β-amyloidosis. • **Harris et al., 2010** - Transsynaptic progression of amyloid-β-induced neuronal dysfunction within the entorhinal-hippocampal network. • **Langer et al., 2011** - Soluble Aβ seeds are potent inducers of cerebral β-amyloid deposition. • **Heilbronner et al., 2013** - Seeded strain-like transmission of β-amyloid morphotypes in APP transgenic mice. • **Ruiz-Riquelme et al., 2018** - Prion-like propagation of β-amyloid aggregates in the absence of APP overexpression. • **Ye et al., 2017**. Aβ seeding potency peaks in the early stages of cerebral β-amyloidosis.
Alzheimer's (Tau)	**IN VITRO (cellular models):** • **Frost et al., 2009** - Propagation of tau misfolding from the outside to the inside of a cell. • **Kfoury et al., 2012** - Trans-cellular propagation of Tau aggregation by fibrillar species. • **Michel et al., 2014** - Extracellular monomeric tau protein is sufficient to initiate the spread of tau protein pathology. • **Woerman et al., 2016** - Tau prions from Alzheimer's disease and chronic traumatic encephalopathy patients propagate in cultured cells. • **Wu et al., 2016** - Neuronal activity enhances tau propagation and tau pathology in vivo. • **Reilly et al., 2017** - Novel human neuronal tau model exhibiting neurofibrillary tangles and transcellular propagation. • **Evans et al., 2018** - Extracellular Monomeric and Aggregated Tau Efficiently Enter Human Neurons through Overlapping but Distinct Pathways. **IN VIVO:** • **Clavaguera et al., 2009** - Transmission and spreading of tauopathy in transgenic mouse brain. • **de Calignon et al., 2012** - Propagation of tau pathology in a model of early Alzheimer's disease. • **Clavaguera et al., 2013** - Brain homogenates from human tauopathies induce tau inclusions in mouse brain. • **Iba et al., 2013** - Synthetic tau fibrils mediate transmission of neurofibrillary tangles in a transgenic mouse model of Alzheimer's-like tauopathy. • **Dujardin et al., 2014** - Neuron-to-neuron wild-type Tau protein transfer through a trans-synaptic mechanism: relevance to sporadic tauopathies. • **Holmes et al., 2014** - Proteopathic tau seeding predicts tauopathy in vivo. 2014. • **Peeraer et al., 2015** - Intracerebral injection of preformed synthetic tau fibrils initiates widespread tauopathy and neuronal loss in the brains of tau transgenic mice. • **Stancu et al., 2015** - Templated misfolding of Tau by prion-like seeding along neuronal connections impairs neuronal network function and associated behavioral outcomes in Tau transgenic mice. • **Fá et al., 2016** - Extracellular Tau Oligomers Produce An Immediate Impairment of LTP and Memory. • **Guo et al., 2016** - Unique pathological tau conformers from Alzheimer's brains transmit tau pathology in nontransgenic mice. • **Jackson et al., 2016** - Short Fibrils Constitute the Major Species of Seed-Competent Tau in the Brains of Mice Transgenic for Human P301S Tau. • **Pickett et al., 2017** - Spread of tau down neural circuits precedes synapse and neuronal loss in the rTgTauEC mouse model of early Alzheimer's disease.

Table 1: continued

Neurodeg. disease	*In vitro, in vivo* studies and findings in patients
Parkinson's (α-syn)	**IN VITRO (cellular models):** • **Lee et al., 2005** - Intravesicular localization and exocytosis of α-synuclein and its aggregates. • **Desplats et al., 2009** - Inclusion formation and neuronal cell death through neuron-to-neuron transmission of α-synuclein. • **Nonaka et al., 2010** - Seeded aggregation and toxicity of α-synuclein and tau: cellular models of neurodegenerative diseases. • **Volpicelli-Daley et al., 2011 -** Exogenous α-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. • **Freundt et al., 2012** - Neuron-to-neuron transmission of α-synuclein fibrils through axonal transport. • **Abounit et al., 2016** - Tunneling nanotubes spread fibrillar α-synuclein by intercellular trafficking of lysosomes. • **Domert et al., 2016** - Aggregated Alpha-Synuclein Transfer Efficiently between Cultured Human Neuron-Like Cells and Localize to Lysosomes. **IN VIVO:** • **Luk et al., 2012a** - Intracerebral inoculation of pathological α-synuclein initiates a rapidly progressive neurodegenerative α-synucleinopathy in mice. • **Luk et al., 2012b** - Pathological α-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. • **Mougenot et al., 2012 -** Prion-like acceleration of a synucleinopathy in a transgenic mouse model. • **Masuda-Suzukake et al., 2013** - Prion-like spreading of pathological α-synuclein in brain. • **Sacino et al., 2014** - Intramuscular injection of α-synuclein induces CNS α-synuclein pathology and a rapid-onset motor phenotype in transgenic mice. • **Bernis et al., 2015** - Prion-like propagation of human brain-derived alpha-synuclein in transgenic mice expressing human wild-type alpha-synuclein. • **Paumier et al., 2015** - Intrastriatal injection of pre-formed mouse α-synuclein fibrils into rats triggers α-synuclein pathology and bilateral nigrostriatal degeneration. • **Abdelmotilib et al., 2017** - α-Synuclein fibril-induced inclusion spread in rats and mice correlates with dopaminergic Neurodegeneration. • **Karampetsou et al., 2017** - Phosphorylated exogenous alpha-synuclein fibrils exacerbate pathology and induce neuronal dysfunction in mice. • **Shimozawa et al., 2017** -Propagation of pathological α-synuclein in marmoset brain. • **Thakur et al., 2017** - Modeling Parkinson's disease pathology by combination of fibril seeds and α-synuclein overexpression in the rat brain. **PATIENTS:** • **Li et al., 2008** - J. Y. et al. Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation • **Kordower et al., 2008a** - Lewy body-like pathology in long term embryonic nigral transplants in Parkinson's disease. Nature Med. 14, 504–506 (2008). • **Kordower et al., 2008b -** Transplanted dopaminergic neurons develop PD pathologic changes: a second case report. • **Li et al., 2010** -. Characterization of Lewy body pathology in 12- and 16-year old intrastriatal mesencephalic grafts surviving in a patient with Parkinson's disease.

Table 1: continued

Neurodeg. disease	*In vitro, in vivo* studies and findings in patients
Huntington's (Huntingtin)	**IN VITRO (cellular models):** • **Ren et al., 2009 -** Cytoplasmic penetration and persistent infection of mammalian cells by polyglutamine aggregates. • **Herrera et al., 2011 -** Visualization of cell-to-cell transmission of mutant huntingtin oligomers. • **Costanzo et al., 2013b -** Transfer of polyglutamine aggregates in neuronal cells occurs in tunneling nanotubes. • **Pecho-Vriesling et al., 2014 -** Transneuronal propagation of mutant huntingtin contributes to non-cell autonomous pathology in neurons. • **Brahic et al. 2016** - Axonal transport and secretion of fibrillar forms of alpha-synuclein, Abeta42 peptide and HTTExon 1. • **Ruiz-Arlandis et al., 2016 -** Binding, internalization and fate of Huntingtin Exon1 fibrillar assemblies in mitotic and non-mitotic neuroblastoma cells. **IN VIVO:** • **Babcock et al., 2015 -** Transcellular spreading of huntingtin aggregates in the Drosophila brain. • **Pearce et al., 2015 -** Prion-like transmission of neuronal huntingtin aggregates to phagocytic glia in the Drosophila brain**.** • **Tan et al., 2015** - Huntington's disease cerebrospinal fluid seeds aggregation of mutant huntingtin. • **Jeon et al., 2016** - Human-to-mouse prion-like propagation of mutant huntingtin protein. **PATIENTS:** • **Cicchetti et al. 2009 -** Neural transplants in patients with Huntington's disease undergo disease-like neuronal degeneration. • **Cicchetti et al., 2014 -** Mutant huntingtin is present in neuronal grafts in Huntington disease patients.
Amyotrophic lateral sclerosis DPR-proteins (*C9orf72*)	**IN VITRO (cellular models):** • **Westergard et al., 2016 -** Cell-to-Cell Transmission of Dipeptide Repeat Proteins Linked to C9orf72-ALS/FTD.
Amyotrophic lateral sclerosis DPR-proteins (TDP-43)	**IN VITRO (cellular models):** • **Nonaka et al., 2013** - Prion-like properties of pathological TDP-43 aggregates from diseased brains. • **Feiler et al., 2015** - TDP-43 is intercellularly transmitted across axon terminals. • **Smethurst et al., 2016** - In vitro prion-like behaviour of TDP-43 in ALS. • **Pokrishevsky et al., 2016** - TDP-43 or FUS-induced misfolded human wild-type SOD1 can propagate intercellularly in a prion-like fashion. • **Ishii et al., 2017** - Formation and spreading of TDP-43 aggregates in cultured neuronal and glial cells demonstrated by time-lapse imaging. **IN VIVO:** • **Porta et al., 2018** - Patient-derived frontotemporal lobar degeneration brain extracts induce formation and spreading of TDP-43 pathology in vivo.
Amyotrophic lateral sclerosis DPR-proteins (SOD-1)	**IN VITRO (cellular models):** • **Munch et al., 2011** - Prion-like propagation of mutant superoxide dismutase-1 misfolding in neuronal cells. • **Basso et al., 2013 -** Mutant copper-zinc superoxide dismutase (SOD1) induces protein secretion pathway alterations and exosome release in astrocytes: implications for disease spreading and motor neuron pathology in amyotrophic lateral sclerosis. • **Pokrishevsky et al., 2017** - Spinal cord homogenates from SOD1 familial amyotrophic lateral sclerosis induce SOD1 aggregation in living cells. **IN VIVO:** • **Ayers 2016** - Prion-like propagation of mutant SOD1 misfolding and motor neuron disease spread along neuroanatomical pathways.

Using the above-mentioned criteria, it can be concluded that the protein aggregates of HTT, Aβ, tau, α-syn, DPR-proteins (C9orf72), TDP-43 and SOD-1 have prion-like properties. However, except for α-syn, these proteins differ from the PrDs for one fundamental characteristic: infectability and transmissibility of the aggregates species between different individuals (i.e. for simple ingestion, blood transfusion and not through complex and artificial experiments *in vivo*) have not been fully demonstrated yet. Therefore, they cannot yet be referred to as prions and are further referred here as *prionoids* (as was proposed by Scheckel and Aguzzi, 2018).

In PD, evidence is accumulating that α-syn aggregates can actually be prions that can spread from the gastrointestinal tract to the brain. The initial idea was grounded by Braak and colleagues by observing that α-syn aggregates appeared in both the brain and the enteric nervous system (ENS) in post-mortem samples of PD patients (Braak et al., 2006). Experimental studies now indeed showed that direct injection of α-syn in ENS of animals can induce pathological features in the brain (including aggregation). The active transport of α-syn occurs through the vagal nerve. Other studies showed similar findings when animal's gut received microbiota from PD donors (Holmqvist et al., 2014; Sampson et al., 2016; Uemura et al., 2018; Manfredsson et al., 2018). These data implies that α-syn, differently from other NDs-associated aggregates, show infectability and transmissibility properties similar to the prion protein, albeit cross-species transmissibility has not yet been shown to occur.

3. Glial cells and brain pathology

Several cell autonomous factors concur to determine neuronal vulnerability (section 2.5). However, our current understanding of brain pathology, which includes the evidence regarding the prion-like properties of NDs-associated aggregates (section 2.6), implies that also non-cell autonomous factors are important contributors of neuronal degeneration.

For a better understanding of NDs and how aggregates drive cell degeneration in NDs, it is therefore impelling to not exclusively focus on neurons and on the cell-autonomous activities of HSPs and PQC in these cells.

The human adult brain contains around 86 billion neurons, but also a similar number of non-neuronal cells that play a key role in the biology of the nervous tissue (Azevedo et al., 2009). Together with the vascular cells, which form the vessels for blood circulation, the main non-neuronal component of the brain is represented by glial cells. Neurons establish several non-cell autonomous interactions with glial cells and such interactions play a key role in the healthy and diseased brain. Our understanding of the nervous system evolved, throughout the years, from a "neuron-centric" view (that considered glia just as the "glue of the brain" with a passive role of structural support) to a more integrated picture, showing a complex network of electrically excitable neurons and electrically non-excitable glial cells with a high level of specialization and cooperativity. Whilst neurons fire action potentials through axons and are responsible for synaptic transmissions, glia have several mutual functional interactions with neurons, mainly aimed to maintain neuronal viability and functionality. Neurons die without glia: glial cells are therefore essential for the homeostasis of the brain (Barres et al., 2008).

Astrocytes, microglia and oligodendrocytes are the three main types of highly specialized glial cells in the mature human central nervous system (CNS) (Barres et al., 2008). In this Thesis, I will focus on astrocytes, the larger glial population in the human brain. An increasing body of evidence shows that astrocytes exert a number of fundamental functions in the healthy CNS, but also suggests that they also play a key role during pathogenesis in NDs (Sofroniew et al., 2010). Activation of astrocytes is a common hallmark of all NDs, including those associated with the presence of protein aggregates (Phatnani and Maniatis, 2015). This state of reactivity is called neuroinflammation and involves not only astrocytes, but also microglia which are CNS-resident macrophage-like cells, primarily deputed to remove cellular debris from the site of brain insult. It is believed that the initial damage-associated reactivity of astrocytes is aimed to positively counteract the disease progression. Nevertheless, a prolonged chronic state of neuroinflammation, which leads to the alteration or loss of the normal functions of astrocytes and gain of new detrimental activities, may rather contribute to the progression of neuronal degeneration (Sofroniew et al., 2010). This is notably observed in Alexander disease, a genetic neurodegenerative disorder that primarily affects astrocytes by a dominant gain-of-function mutation of the *GFAP* gene (Olabarria et al., 2017). Together, these observations suggest that the biology of NDs may not be exclusively ascribed to protein aggregation and PQC anti-aggregation activities in neurons (cell autonomous components of pathogenesis), but is also influenced by the positive and/or negative, molecular and functional interactions of neurons with the tissue environment and particularly with the other brain cells like astrocytes (non-cell autonomous components of pathogenesis). In the next sections, I will discuss the capacity of astrocytes to protect neurons against NDs-associated aggregates in a non-cell autonomous manner. In particular, I will focus on the observations that the molecular changes detected in reactive astrocytes during NDs include the up-regulation of certain HSPs. The functional implications of such HSPs up-regulation for the progression of neuronal degeneration have not yet been investigated. However, the peculiar role of astrocytes in the brain and the established key role of HSPs against protein aggregation and aggregate toxicity suggest that the HSPs astrocytic response in astrocytes may have a functional protective significance in these diseases.

3.1: The role of astrocytes in the healthy brain

For their homeostasis, neurons depend on astrocytes, with which they establish multiple functional interactions (Belanger et al., 2011). Astrocytes are the larger glial population in the brain and provide structural support, tiling the entire CNS. The most striking feature of astrocytes is represented by their many long processes, that give their typical star-shape and are required for contacting neurons in different cell regions including synapses (synaptic processes) (Sofroniew et al., 2010).

Like neurons, astrocytes can show a high level of heterogeneity in morphology and function. Based on their anatomical position in the brain tissue and on morphology, they are classified as protoplasmic, when found in the gray matter and showing finely branching processes, or fibrous when located in the white matter and showing long fiber-like processes (Cajal, 1909).

Interestingly, astrocytes are “territorial” cells: with its processes, a single astrocyte can contact many contiguous neurons and synapses, covering a “territory” (also defined as “domain”), with little

overlap between neighboring astrocytes (Nedergaard et al., 2003; Halassa et al., 2007b). These domains are functionally interconnected through connexins-gap junctions (Giaume et al., 2010), which permit to astrocytes to communicate through signaling waves of calcium ions and to exchange molecules each other. Such interconnection of several astrocytes forms a glial syncytium, a multicellular network that embraces the neuronal circuits, with which they have several interactions (Nedergard et al., 2003; Obhereim et al., 2006).

The functional interactions of astrocytes with neurons include the following:

1. Function in the multi-partite synapse:

An example of the cellular specialization in CNS is the multi-partite synapse, a site of interplay between astrocytes with the pre- and post-synaptic neurons (Araque et al., 1999; Halassa et al., 2007a; Perea et al., 2009). Here, astrocytes exert essential functions by maintaining the fluid, ions, pH and transmitter homeostasis. Astrocytic processes are in fact rich in aquaporin water channels (e.g. AQP4) and transporters for ions (e.g. K^+, Na^+, H^+ and bicarbonate) (Simard et al., 2004; Seifert et al., 2006; Obara et al., 2008) and neurotransmitters (e.g. glutamate, GABA and glycine) (Sattler et al.,2006; Seifert et al., 2006,). The neurotransmitters released in the synaptic cleft by the pre-synaptic neuron can be taken up by astrocytes and are then enzymatically converted into precursors and recycled back to synapse for reconversion into active transmitters. A typical example of this neurotransmitter release and recycle between neurons and astrocytes is the glutamate-glutamine cycle (Bak et al., 2006; Mckenna et al., 2007). As also previously discussed, any imbalance in pH and levels of neurotransmitters and ions in the synaptic cleft are detrimental for neurons and may play an important role in many NDs (e.g. in the process of excitotoxicity). Therefore, the buffering capacity of astrocytes in the multi-partite synapse is crucial to maintain the neuronal fitness. This can impact on protein aggregation in the over-activated post-synaptic neurons because the resulting intracellular ionic imbalance (i.e. Ca^{2+}) can trigger the unfolding of disease-associated proteins or cause their cleavage by proteases (section 2.4).

2. Support to neuronal plasticity:

Astrocytes release different molecules into the extracellular space, some of which can activate neurons, such as glio-transmitters (e.g. glutamate, ATP, adenosine, GABA and D-serine) (Nedergaard et al., 2003; Volterra et al., 2005; Halassa et al., 2007a; Perea et al., 2009), growth factors (Banker et al., 1980; Sofroniew et al., 2010; Belanger et al., 2011,), neuroactive steroids (e.g. estradiol, progesterone) (Garcia-Segura et al., 2006) and matrix-associated proteins (e.g. thrombospondins; Christopherson et al., 2005). The molecular exchanges with neurons are fundamental for the adaptive plasticity of CNS, implying a role of astrocytes in controlling dendritic and axonal arborization, synapses connections (synaptogenesis and synaptic pruning), receptors composition at synaptic cleft and neurotransmitters regulation (Sofroniew et al., 2010). Adaptive plasticity has an important role not only in developing CNS, but also during functional regeneration of neurons after a damage, therefore with implications also in NDs. As discussed before, NDs-aggregates in neurons may exert a toxic activity on synaptic functionality and structure (section 2.4), which could be eventually exacerbated by the loss of astrocytic functions.

3. Metabolic cooperation:

The CNS is high energy-consuming and neurons have a very high energy requirement, making them extremely dependent upon the supply of energy substrates from the blood circulation. Astrocytes strictly monitor the trafficking of energy metabolites (such as glucose and lactate), being in contact with both neurons and the blood vessels. Although neurons and astrocytes show a different metabolism, they are also highly cooperative (Belanger et al., 2011). Neurons have a high oxidative metabolism (Lebon et al., 2002; Itho et al., 2003; Boozier-Sore et al., 2006; Boumezbeur et al., 2010a) efficiently use lactate as primary source of energy substrate (Schurr et al., 1997; Bouzier et al., 2000; Qu et al., 2000; Itoh et al., 2003; Serres et al., 2005; Bouzier-Sore et al., 2006; Boumezbeur et al., 2010b) and are low glycolytic (Almeida et al., 2004; Herrero et al., 2009; Bolanos et al., 2010) (a specialization that protects them from oxidative stress). Differently, astrocytes have low oxidative metabolism, take up glucose, are high glycolytic and generate glycogen as energy reserve (Itho et al., 2003; Herrero et al., 2009; Bittner et al., 2010). Importantly, astrocytes transfer energy metabolites to neurons by responding to any increase of neuronal activity and energy demand. To sense this, astrocytes monitor the level of extracellular glutamate released by neurons and increase glycolysis and lactate production. The energy metabolites produced by astrocytes are then released into the extracellular space and taken up and used by neurons for ATP production (Belanger et al., 2011). Another important example of astrocytes-neurons cooperation concerns the cholesterol metabolism: brain cells are separated from the periphery due to the Blood-Brain-Barrier and they cannot receive the cholesterol from the blood. Therefore, astrocytes synthetize *de novo* the cholesterol pool in the brain and, similarly to what happens for energy metabolites, they transfer it to neurons, by which it is used for membranes generation and synthesis of derivative molecules (Priefeger et al., 2011). During NDs, metabolic processes for ATP production, that increase the production of ROS, might boost protein aggregation in neuronal and non-neuronal cells in the brain. Interestingly, it has been observed that in HD, mutant aggregate species of HTT are capable to reduce the cholesterol biosynthesis by interfering with the transcription regulatory pathway of SREBP (sterol responsive element-binding protein). This might have detrimental effects on the astrocytic capability to *de novo* produce the cholesterol pool in the brain, for being used by neurons to build up membranes and other important functions (Valenza et al., 2011).

4. Neuronal protection from oxidative damage:

ROS, generated with the cellular oxidative metabolism, can stress and damage neurons. As previously discussed, ROS can also directly cause covalent modifications in proteins, an important trigger in the process of aggregation (section 2.4). Astrocytes release anti-oxidant molecules and ROS-scavengers (e.g. glutathione) to neurons, providing a non-cell autonomous protection from oxidative damage (Chen et al., 2001; Shih et al., 2003). As explained before, a loss of astrocytic function in producing and releasing such scavengers might promote the accumulation on ROS and consequentially protein aggregation in neurons (section 2.4).

5. Formation of the Blood-Brain-Barrier (BBB):

The vascular endfeet of astrocytes cover all the vascular surfaces in the brain. Together with the basal lamina, the capillary endothelial cells, and the perivascular pericytes, astrocytes form the BBB, an interface that selects and controls the movement of molecules and cells between blood and the CNS extracellular space, providing an essential protection to neurons, also from all those factors

that can trigger protein aggregation. Through these contacts, astrocytes release molecular mediators (e.g. prostaglandins, nitric oxide and arachidonic acid) that control the CNS blood vessel diameter and blood flow. In response to increased neuronal activity, astrocytes can also control and increase the delivery of oxygen and nutrients from blood to the active brain region. These several functions of BBB might also have a great impact in NDs pathogenesis: a significant role of structural and functional changes of BBB observed in NDs suggests that BBB alteration might contribute to the aetiology of these disorders. However, as similar BBB changes are observed in many different NDs, it might be likely that the barrier dysfunctions are consequence of the neurodegeneration. BBB changes are an important component of neuroinflammation and they can alter BBB integrity, transport and regulatory functions (e.g. as signaling interface), enhancing the entering of immune cells from the periphery into the nervous tissue (Carvey et al., 2009).

Below, I will next describe whether or not neuroinflammation is detrimental or beneficial in NDs, in particular focusing on the role of astrocytes.

3.2: The role of astrocytes in the brain during disease and implications in NDs

Neuroinflammation used to be considered as an unregulated glial response to brain diseases that might cause neurotoxicity and exacerbate the pathological conditions. This had led to the idea that inhibition of neuroinflammation might be used as a therapeutic strategy against NDs. More recently, however, neuroinflammation is no longer considered an all-or-none uncontrolled response, but a fine-tuned regulated continuum of progressive changes in glial cells. These changes may be initially positive for neurons and aim to counteract the disease process, being part of the normal protective functions of glia. Nevertheless, a prolonged chronic state of neuroinflammation, which might occur in NDs, changes glial activities through alteration or loss of their normal functions and gain of detrimental functions which may finally contribute to degenerate neurons in a non-cell autonomous way (Sofroniew et al., 2009; Sofroniew et al., 2010).

Together with protein aggregation, neuroinflammation represents another common hallmark of NDs. In this state, reactive astrocytes and microglia, the major glial mediators of the immune response in CNS, respond to the pathological condition and to the tissue damage through a spectrum of molecular, functional and cellular changes during the disease progression, which mainly aim to restore the homeostasis of the tissue (Ben Haim et al., 2015; Heneka et al., 2014).
Astrocytes, due to their several functional interactions with neurons during health and disease, have a fundamental role during neuroinflammation and in the pathogenesis of NDs associated with protein aggregates. In such context, two open questions are currently under investigation: 1 to) understand how astrocytes react to the degeneration of neurons and other brain cells during the disease; 2) to establish how the cellular and functional changes that characterize the astrocytic reactivity can influence the neuronal fitness during the pathology in a non-cell autonomous manner.

In NDs, reactive astrocytes are generally found in those CNS regions that are more vulnerable to the respective protein aggregation and neurodegeneration:

- ✓ In HD brains, reactive astrocytes are primarily observed in caudate and putamen during the early stage and later also in the motor cortex, globus pallidus, thalamus and hippocampus (Vonsattel et al., 1985; Faideau et al., 2010).
- ✓ In PD brains, they are found in substantia nigra (Forno et al., 1992; Damier et al., 1993).
- ✓ In AD patients, reactive astrocytes appear before clinical symptoms and are initially found in the hippocampus and entorhinal cortex and later in the temporal, frontal and parietal lobes. Notably, they are also found around the extracellular plaques of β-amyloid, although not in all cases (Simpson et al., 2010; Carter et al., 2012,).
- ✓ Also in ALS, reactive astrocytes are observed in the vulnerable regions and notably before the manifestation of motor symptoms (Maragakis et al., 2006; Philips et al., 2011).

Importantly, the reactive astrocytes may already appear in the early pre-symptomatic stage of the disease (Halm et al., 2015), supporting the idea that the initial astrocytic response might be a protective process to counteract the progression of neurodegeneration.

In NDs, reactive astrocytes are hypertrophic, they overexpress GFAP, and reorganize and polarize their processes toward the site of neurodegeneration (Sofroniew et al., 2009; Ben Haim et al., 2015). Astrogliosis - the increased proliferation of astrocytes - is a component of neuroinflammation. Data from disease rodent models indicate that the magnitude of astrogliosis is very limited during the early phase of NDs, compared to the reaction observed after acute brain injury (Khamphuis et al., 2012; Sirko et al., 2013). This again supports the idea that the astrocytic reactivity is not always characterized by an intense cell division that ultimately aims to merely form a glial scar, but rather is a regulated set of progressive and functional changes that strictly depend by the type and extent of brain damage.

During neuroinflammation, astrocytes proceed through different levels of reactivity. In the healthy brain, astrocytes show non-overlapping domains and do not express high levels of GFAP. During a mild-moderate neuroinflammation, which may characterize the early phase of any brain disease, the structural organization of astrocytes is maintained and they incur molecular changes and hypertrophy that are potentially reversible if the origin of the damaged is resolved. Instead, if the insult continues and degeneration of neurons persists, such as in chronic and progressive brain diseases (e.g. NDs), the cellular and functional changes in astrocytes progress alongside with the brain damage and their structural domain-based organization is disrupted. The process may end with the formation of glial scars along the border of the damage site: this structure is mainly formed by astrocytes and other glial cells with high deposition of dense collagenous extracellular matrix. The state of glial scars is considered an irreversible condition of astrocytes and it is thought to primarily serve as a barrier between the healthy tissue and the area of damage and inflammation (Sofroniew et al., 2010).

Perturbations in the homeostasis of the brain are sensed by astrocytes through plasma membrane-receptors, which are responsive to molecules released by other cells in the damaged tissue. Such

signals may be released by all the cells involved in the pathological process, including affected neurons and the glia in the site of damage and degeneration. Astrocytes, together with microglia, are the main mediators of the immune response, and once activated they can signal to other cells, propagating the neuroinflammation state throughout the brain (Buffo et al., 2010; Burda et al., 2014; Kigerl et al., 2014). The spectrum of released molecules can vary, depending on the origin of the damage (e.g. NDs, infections, trauma, stroke) and can differ in case of acute injury (such as in a stroke) or chronic slow degeneration of cells (such as in NDs). Although the exact molecular triggers of astrocyte reactivity for each specific NDs are still under investigation, several of them have been identified. The spectrum of signals, which characterizes the neuroinflammation during NDs includes polypeptide growth factors (e.g. CNTF, BDNF, GDNF, NT3, EGF, bFGF, IGF1) (Nieto-Sampedro et al., 1990; Scharr et al., 1993; Schwartz et al., 1994; Kahn et al., 1997; Hinks et al., 1999; Messermsmith et al., 2000; Smith et al., 2001; Chen et al., 2006b; Escartin et al., 2007) cytokines (e.g. interleukins, TNFα, INF-γ, TGFβ) (Yong et al., 1991; Chiang et al., 1994; Korderk et al., 1996; Giulian et al., 1998; Campbell et al., 2001; Swartz et al., 2001; Lin et al., 2006a), neurotransmitters (e.g. glutamate) (Bekar et al., 2008), ROS, nitric oxide (Swanson et al., 2004) and other molecules associated with unpaired metabolism (e.g. $NH4^+$) (Norenberg et al., 2009).

Receptors for these signals are present on the plasma membrane of astrocytes and, once activated, they trigger intracellular signaling cascades (e.g. JAK/STAT-, NF-κB-, calcineurin- and MAPK-pathways) that initiate those molecular changes that lead to the cellular and functional changes in astrocytes during neuroinflammation (Buffo et al., 2010; Ben Haim et al., 2015). As previously said, astrocytes monitor each synapse in the brain, and it is estimated that the processes of one astrocyte can contact over 100.000 synapses (Araque et al., 1999; Halassa et al., 2007a; Perea et al., 2009). Astrocytes are therefore well positioned at the tripartite synapses to rapidly and efficiently detect, through these receptors, any abnormal neuronal activity (e.g. release of ROS and $NH4^+$), during the pathological processes associated with NDs. The presence of such signals in the extracellular space may justify the presence of reactive astrocytes also in brain regions which are relatively distant from the site of neuronal degeneration.

In the early phase of NDs, the activity of reactive astrocytes may be beneficial to and protective for neurons (Silver et al., 2004). In addition to the previously mentioned functions, reactive astrocytes exert several new important activities that support neuronal viability and counteract the disease. Important examples of these are:

- The release of neuroprotective molecules such as cytokines and growth factors (e.g. CNTF, BDNF, NGF and FGF) (Nieto-Sampedro et al., 1990; Scharr et al., 1993; Schwartz et al., 1994; Kahn et al., 1997; Hinks et al., 1999; Messermsmith et al., 2000; Smith et al., 2001; Chen et al., 2006b; Escartin et al., 2007; Vargas et al., 2008).
- The release of anti-oxidant molecules that block ROS toxicity (e.g. glutathione, ascorbic acid) (Chen et al., 2001; Shih et al., 2003).
- The re-uptake of excitatory neurotransmitters, like glutamate, from the synaptic cleft (therefore counteracting excitotoxicity) (Rothstein et al., 1996; Bush et al., 1999).
  The repair and maintenance of the BBB (Carvey et al., 2009).

- The restriction of inflammatory cells at the site of damage (via the formation of the glial scar) (Bush et al., 1999; Faulkner et al., 2004; Myer et al., 2006; Okada et al., 2006; Herrmann et al.,2008; Voskul et al., 2009;).

It is important to note that the commonality of the changes in reactive astrocytes (i.e. release of the same neuroprotective molecules) that is observed among different NDs (i.e. different types of neurons, different protein aggregates, different aggregate location), strongly supports the hypothesis that such changes are a consequence and not a cause of the disease. Protein aggregation primarily in neurons and neuronal damage trigger astrocytic reactivity and neuroinflammation in the site of damage.

Due to the chronic nature of NDs and the progressive accumulation of the neuronal damage over time, (reactive) astrocyte may lose their normal functions of support and protection toward neurons (see previous section) and acquire new toxic and detrimental functions (Sofroniew et al., 2010; Ben Haim et al., 2015). In the final step, astrocytes may even die, further aggravating the pathology. In addition to the loss of their normal functions, chronic reactive astrocytes may produce and release abnormal cytotoxic levels of inflammation-associated signals (Brambilla et al., 2005; Brambilla et al., 2009), ROS (Hamby et al., 2006) and other toxic metabolites like glutamate (Takano et al., 2005) that can exacerbate the disease condition. Moreover, although fundamental to isolate the site of damage, the formation of the glial scar in the late phase is considered detrimental for brain regeneration, because it also inhibits the axon regeneration and the formation of new synaptic connections (Silver et al., 2004).

The key role of protein aggregates in NDs and the presence of reactive astrocytes in the brain of the patients suggest that aggregates might trigger astrocyte reactivity, as an important component of ND pathology. But inversely, does this reaction also serves to handle aggregates? This question is even more relevant in the context of the prion-like behavior of many aggregating ND proteins. So, what do we know about the presence of aggregates in astrocytes? And what is their sensitivity to aggregates. How do they handle them and may this handling play a role in the ethology of the disease? These questions are addressed in the next sections.

3.3: Astrocytes and NDs-associated aggregates

Astrocytes with intracellular aggregates are indeed observed in most NDs, mainly in the affected brain regions where protein aggregation in neurons also occurs. However, the frequency of astrocytes with aggregates is much lower compared to the frequency of neurons with intracellular aggregates (considering the same affected brain region) (Miller et al., 2004; Maragakis et al., 2006; Jansen et al., 2014; Phatnani et al., 2015). This is also observed in PolyQ diseases, including HD (Jansen et al., 2017).

The reason for this is unknown, but one could speculate the following one or more options:

- Astrocytes are mitotic cells and, differently from neurons, they can be regenerated in the adult brain; during division and differentiation, cells might then be capable of asymmetric partitioning of

aggregates, an important protective survival strategy conserved from bacteria to yeast and dividing (stem) cells in metazoan (section 2.1).

- Astrocytes are resistant to intrinsic triggers for cell autonomous aggregation. They are shown to have a low oxidative metabolism (Belanger et al., 2011) and higher levels of endogenous molecules (such as ROS-scavengers) that might protect them from protein damage (Sofroniew et al., 2010). Moreover, they might have better PQC to prevent or handle/degrade the aggregates (sections 3.3.1 and 3.4).

- Astrocytes do not take up aggregates and thus do not take part in the prion-like processes; inversely they do take up the prion-like aggregates, but have better capacity to handle them compared to neurons (see 3.3.2 and 3.4).

3.3.1 Aggregates intracellularly formed in astrocytes

Several aggregation-prone proteins responsible for different NDs are ubiquitously expressed in all the cells of the body. For example, the *HTT* human gene is regulated by a promoter with a widespread constitutive expression with little regulation. HTT is therefore ubiquitously expressed in neuronal and non-neuronal cells (Zuccato et al., 2010).

Due to the ubiquitous expression of toxic proteins in the brain of the patients, the specific effects of ND-associated protein aggregation in astrocytes cannot be easily discriminated and characterized through the post-mortem analysis of these samples. Such characterization is also not possible using *in vivo* disease models with ubiquitous promoters.

Only by using *in vivo* models in which the toxic protein is exclusively expressed in astrocytes, it is possible to evaluate its impact on the overall homeostasis of the brain. The studies summarized in Table 2 used the promoter of the *GFAP* gene for the selective astrocytic expression of transgenes in rodent models. The expression of different NDs-associated proteins in astrocytes was found to cause formation of intracellular aggregates, alteration of their neuronal-supporting functions (e.g. activity of glutamate EAAT transporters, BBB integrity), triggered astrocytic reactivity and induced neuroinflammation. Interestingly, another important and common finding in these models is the degeneration of neurons close to the site of reactive, aggregates-containing astrocytes. Phenotypically, animals show age-dependent neurological symptoms (e.g. motor impairments) and reduced lifespan, meaning that protein aggregation is detrimental for astrocyte fitness and/or functionality and can trigger their reactivity (cell-autonomously). The affected astrocytes next can lead to neurodegeneration, in a non-cell autonomous manner (Forman et al., 2005; Dabir et al., 2006; Bradford et al., 2009; Bradford et al., 2010; Gu et al., 2010; Tong et al., 2013).

***Table 2*: Expression of pathogenic proteins in astrocytes leads to protein aggregation and neurodegeneration in in vivo models of NDs**. *Studies are summarized that illustrate the phenotype of rodent models expressing different NDs-associated proteins exclusively in astrocytes. Type of toxic proteins, transgene sequence, promoter for expression and main findings are reported.*

Toxic Protein	Construct	Promoter	Method	Phenotype	Ref.
HTT	N-terminal human HTT (1–208 amino acids) with 160Q	human *GFAP* promoter	Overexpression in Astrocytes	Reactive astrocytes (increased GFAP) and neuroinflammation, altered astrocytic functionality, PolyQ HTT aggregates in astrocytes, no clear sign of cellular degeneration, age-dependent neurological symptoms and reduced lifespan.	**Bradford J et al., 2009.**
HTT	N-terminal human HTT (1–171 amino acids) with 82Q	human *GFAP* promoter	Overexpression in Astrocytes		**Bradford J et al., 2010.**
α-syn	mutant human A53T α-syn	human *GFAP* promoter	Overexpression in Astrocytes	Reactive astrocytes (increased GFAP) and neuroinflammation, altered astrocytic functionality, A53T α-syn aggregates in brain, neuronal degeneration, age-dependent neurological symptoms and reduced lifespan.	**Gu et al., 2010.**
Tau	human Tau	human *GFAP* promoter	Overexpression in Astrocytes	Reactive astrocytes (increased GFAP) and neuroinflammation(with BBB disruption), altered astrocytic functionality, Tau aggregates in astrocytes, neuronal degeneration	**Forman et al., 2005.**
Tau	human Tau, mutation P301L (FTDP-17)	human *GFAP* promoter	Overexpression in Astrocytes	Reactive astrocytes (increased GFAP) and neuroinflammation, altered astrocytic functionality, Tau aggregates in astrocytes, motor impairments.	**Dabir et al., 2006.**
TDP43	Human TDP43, mutation M337V	human *GFAP* promoter	Overexpression in Astrocytes	Reactive astrocytes (increased GFAP), altered astrocytic functionality, ubiquitin inclusions in astrocytes, neuronal degeneration, motor impairments.	**Tong et al., 2013.**

All together, these studies show that astrocytes are intrinsically sensitive to aggregates and that this may lead to their functional impairment. These can subsequently lead to signs of neurodegeneration. Nonetheless, it is important to emphasize that these data do not sustain the idea that neurodegeneration is exclusively due to protein aggregation in astrocytes, as the selective expression of the same disease-causing proteins in neurons (and not in astrocytes) also leads to a phenotype characterized by protein aggregation and neurodegeneration.

3.3.2 Prionoids spreading into astrocytes

As mentioned above (section 2.6), NDs-associated aggregates in HD (HTT), AD (A-Beta, tau), PD (α-syn) and ALS (DPR proteins, TDP43 and SOD1) have prion-like characteristics. Besides neuron-to-neuron transmission, spreading may also involve neuron-to-astrocyte transmission. In fact, one

could hypothesize that a fraction of NDs-associated aggregates observed in astrocytes are not originated *in situ,* but they might be prionoids that have entered into the astrocytes in a prion-like manner. Even more so, by taking up aggregates, astrocytes may counteract spreading to neurons and hence could slow down the process of degeneration. Below, I summarize some speculations in support of such scenarios.

First, as much as any other cell types, astrocytes may be "vulnerable" to the entry of prionoids via passive uptake (i.e. by disruption of the plasma membrane), because this is an intrinsic property of the aggregates and does not seem to depend by the type of acceptor cells (Butterfield et al., 2010).

Second, it has been shown that the active entry of prionoids is mediated by mechanisms which are also observed in astrocytes: notable examples are receptor-mediated endocytosis or the transport via tunneling nanotubes (Davis et al., 2008 and Discussion of this Thesis).

Whereas most *in vitro* studies have used various human and rodent (neuronal) cell lines to investigate transmission of prionoids (table 1), some have done the same for astrocytes. Accumulating evidence comes from *in vitro* studies on PD that have shown that α-syn aggregate species released from neuronal cells can enter in astrocytes (via either endocytosis or the formation of tunneling nanotubes) (Lee et al., 2010; Braidy et al., 2013; Lindstrom et al., 2017; Rostami et al., 2017). Data *in vitro* (Danzer et al., 2007; Hansen et al., 2011) and *in vivo* (Angot et al., 2012) have shown that these α-syn prionoids have seeding properties in several types of recipient cells (although this still needs to be confirmed to happen also in astrocytes).

Similar data have been suggested for aggregates of dipeptide repeat proteins linked to the *C9orf72* (Westergard et al., 2016).

Alongside with these *in vitro* studies, investigations conducted in rodent and insect models permitted to further substantiate the possibility of *in vivo* spreading of prionoids into astrocytes. Luk and colleagues (Luk et al., 2012) have shown that a single injection of synthetic α-syn fibrils in the brain of α-syn transgenic mouse can initiate cell-to-cell transmission and dramatically accelerate both the formation of PD-associated intracellular aggregates and the onset of disease; interestingly, they also found evidences that astrocytes -- among other cells in the injected mouse brain -- were acceptor for these aggregates.

In another study, exosomes containing α-syn, isolated from the frontal cortex of patients with Lewy bodies dementia and injected in the hippocampus of wild-type mice, caused α-syn aggregate formation localized in both neurons and astrocytes (Ngolab et al., 2017).

Similarly, the intracerebral inoculation in mice with brain homogenates from AD patients induced tau-like pathology, tau aggregation and spreading with astrocytes being acceptor cells for the tau protein (Clavaguera et al., 2013; Boluda et al., 2015).

Another approach to study *in vivo* the spreading of aggregates is the expression of the transgenic toxic protein in a specific population of brain cells (donors) and verify if such protein can be later found in another cell type (acceptors). Using such an approach, Pearce and colleagues have shown that HTT expressed in *D. melanogaster* neurons can spread to glial cells. They showed that glia requires the scavenger receptor draper (homolog of the human gene *MEGF11*) and uses a phagocytic engulfment machinery to accept the HTT prionoids (Pearce et al., 2015).

Similarly, de Calignon and colleagues observed that mice expressing mutant tau in a specific population of neurons of the entorhinal cortex found tau aggregates to appear in astrocytes at 24 months of age but not at earlier time points, indicating that tau is likely released from neurons and taken up by astrocytes, as the axons degenerate (de Calignon et al., 2012).

Inversely, some studies have shown that prionoids can be released from astrocytes and enter neurons. For example, astrocytes generated from ALS patients can release exosomes containing mutant SOD-1 that are toxic to co-cultured neurons (Haidet-Phillips et al., 2011; Basso et al., 2013). Similarly, stem cells-derived (rat) astrocytes overexpressing TDP43 can release TDP43 prionoids, which subsequently can be internalized in co-cultured neuronal cells (Ishii et al., 2017).

These *in vitro* and *in vivo* studies highlight that astrocytes could play different roles in the biology of ND as possible acceptor cells of prionoids, hereby functioning as a matrix-barrier in slowing down the spreading of toxic species to neurons. However, astrocytes may have a limited capability to do so, e.g. due to the intrinsic toxicity of the accumulated aggregates. Hereby, their loss may contribute to the disease, either by loss of their neuronal support functions and/or by now becoming donor cells of the prionoids. In such perspective, boosting the capacity of astrocytes to take up and sustain their viability by properly handling the toxic prionoids might have a therapeutical value.

In the next section, I will summarize the existing knowledge on how astrocytes react to the presence of the aggregates and which factors might make them more resistant to such aggregates.

3.4: Astrocyte response to NDs-associated aggregates: intrinsic resistance, protein quality control and expression of HSPs

The less frequent presence of aggregates in astrocytes compared to neurons (as described in section 3.3) may be due to differences in protein quality control, and expression/activity of HSPs. In the next sections I will explore such differences, highlighting some key aspects that may be crucial in the process of neurodegeneration.

3.4.1- Differences between neurons and astrocytes in the UPS

Tydlacka and colleagues reported that UPS is less active in neurons in comparison to white matter glia, suggesting that the glial resistance to protein aggregation might be due to a higher activity of the UPS in sustaining protein degradation and turn-over (Tydlaka et al., 2008; Jansen et al., 2014). Interestingly, it has been observed that during ND-associated neuroinflammation, the immunoproteasome is induced in glia (particularly in astrocytes and microglia) and neurons. In fact, the immunoproteasome induction is a common hallmark in AD (tau) (Mishto et al., 2006; Orre et

al., 2013) and ALS (SOD-1) (Puttahaparty et al., 2007; Cheroni et al., 2009). Recent data have shown the same in PD (α-syn) (Ugras et al., 2018). In HD (HTT), the induction of the immunoproteasome is observed in neurons, but no data have shown the same in glia so far (Díaz-Hernández et al., 2003).

The expression of the immuno-subunits is induced by factors released by the cells during neuroinflammation (e.g. IFNγ). In the immunoproteasome, certain proteasomal subunits are substituted by specific immuno-subunits that change the cleavage capability of the proteasome (Jansen et al., 2014) Whether such substitutions improve the proteasomal activity in degrading ND-associated toxic proteins and, therefore, in counteracting aggregation is yet unclear. However, some recent data support such assumption: Ugras and colleagues (Ugras et al., 2018) have shown that immunoproteasome is capable of degrading α-syn fibrils *in vitro* in cell-free conditions. Next, they confirmed that α-syn fibrils are capable to enter in cells and induce a neuroinflammation response, as previously shown (Lee et al., 2010). Finally they showed that the inhibition of the immunoproteasome increases intracellular accumulation of the aggregates, suggesting a neuroprotective role of the immunoproteasome in response to α-syn aggregation.

3.4.2- Heat shock response in neurons and astrocytes and HSPs upregulation in astrocytes during disease

As previously explained (section 2.1), HSPs can be constitutively expressed or stress-induced.

In different tissues and in cells of the same tissue, the HSR can vary in speed of transcript generation and fold increase in HSPs levels (Sala et al., 2017). Importantly, different cells within the same tissue can have different capacities to induce the HSR. Interestingly, such difference has been also observed for cells in the brain (San Gil et al., 2017).

In one of the first *in vitro* studies where cultured cortical neurons and astrocytes were compared for induction of mRNA and protein levels of HSPA/HSP70 after heat shock, astrocytes showed a faster and stronger response than neurons (Nishimura et al., 1991).

In line, several *in vivo* studies (Manzerra et al., 1992; Nishimura et al., 1996; Manzerra et al., 1997; Krueger et al., 1999; Pavlik et al., 2007; Oza et al., 2007; Yang et al., 2008) have shown that in rodent models treated with hyperthermia or other stress conditions (i.e. induced ischemia), neurons do not induce HSPA/HSP70 expression after exposure to stress conditions, whereas surrounding astrocytes do.

These studies indicate that neurons under different conditions of stress have lower intrinsic capacities to mount the HSR as compared to astrocytes. As the HSR contributes to maintain protein homeostasis, this difference might be one possible factor (but not the unique) that explains why neurons are more affected by protein aggregation in neurodegenerative diseases and why astrocytes are more capable to cope protein aggregation.

However**,** several studies *in vitro* and *in vivo* revealed that several pathogenic proteins (i.e. PolyQ-HTT and SCA3, SOD-1 and TDP43) do not activate HSF-1, at least not before a massive aggregation has already occurred (Tagawa et al., 2007; Chafekar et al., 2012; Bersuker et al., 2013; Seidel et al.,

2016; San Gil et al., 2017), meaning that the capacity to activate the HSR may not be a prime factor responsible for the differential sensitivity of astrocytes and neurons to the initiation of aggregation of the ND-associated proteins.

In fact, HSPs-overexpression screens from our lab have indicated that not always the HSR-regulated HSPs are the best suppressors of aggregation, in particular not for PolyQ proteins (Kakkar et al., 2014). Importantly, it has been established that the human chaperonome consists of many other HSPs that are not regulated (or only marginally) by the HSR and HSF-1, but instead by "non-canonical" regulatory pathways that take place in chronic stress conditions (such as during NDs) (Hageman et al., 2009; Kakkar et al., 2014; Mahat et al., 2016; Solis et al., 2016; Neueder et al., 2017).

Some of these non-canonical and less investigated HSPs show protection in *in vivo* models of protein aggregation diseases. The protective activity of these HSPs differs between different disease-associated proteins, suggesting that each type of aggregate is biochemically distinct and requires specific HSPs to prevent its formation or target it for degradation. This also suggest that no generic explanation can be given in terms of HSP expression that would explain the relative resistance of astrocytes to aggregation.

Table 3: section A (HSPAs, DNAJs, HSPCs and chaperonins) and section B (small HSPs): HSPs expression in astrocytes in the brain of NDs patients or in vivo animal disease model*. Data are grouped per HSPs families and per each HSP member. Disease, type of analyzed sample, method of analysis, and main findings are reported. (Abbreviations: MSA: multiple system atrophy; α-synP: α-Synucleinopathy; tauP: sporadic and familiar tauopathies; FTLD: Frontotemporal lobar degeneration; IHC= Immunohistochemistry; MA=microarray analysis; RT qPCR= Real Time qPCR; ISH= In situ hybridization).*

HSP	NDs	Samples and Meth.	Meth. HSPs in astrocytes: main findings	Ref.
HSPA (HSP70)				
HSPA1A (HSP70-1; HSP72; HSPA1)	MSA, α-SynP (α-Syn)	In vivo, human IHC	Increased HSPA1A/HSP70 in MSA brain. Many reactive astrocytes in the area of neurodegeneration were positive for HSPA1A/HSP70. Co-localization of HSPA1A/HSP70 with glial cytoplasmic inclusions.	**Kawamoto et al., 2007**
HSPA9 (GRP75; HSPA9B; MOT; MOT2; PBP74; mot-2; mortalin)	PD	In vivo, human IHC	HSPA9/mortalin, normally expressed in astrocytes, was reduced in the astrocytes of PD patients located in affected brain regions.	**Cook et al., 2016**
DNAJ (HSP40)				
DNAJB6 (Mrj; mDj4)	PD	In vivo, human RT-qPCR ISH IHC	DNAJB6 is a component of Lewy bodies in both PD substantia nigra and PD cortex. DNAJB6 is strongly up-regulated in parkinsonian astrocytes, and located especially at the astrocytic endfeet.	**Durrenberg et al., 2009** **Michael et al., 2011**
HSPCs (HSP90)				
HSPC1 (HSP90AA1; HSPN; LAP2; HSP86; HSPC1; HSPCA; HSP89; HSP90A; HSP90N; HSPCAL1; HSPCAL4; FLJ31884)	MSA (PD-like α-synP)	In vivo, human IHC	HSPC1/HSP90 is expressed in glial cells (including astrocytes) and co-localizes with glial α-synuclein inclusions	**Chiba et al., 2012**
	PD (and other α-SynP)	In vivo, human IHC	HSPC1/HSP90 is expressed in glial cells (including astrocytes) and often co-localizes with glial α-synuclein inclusions ● *See also for HSPB5 same study*	**Uryu et al., 2006**
Chaperonins				
HSPD1 (HSP60; GroEL)	PD (and other α-SynP)	In vivo, human IHC	HSPD1/HSP60 is expressed in glial cells (including astrocytes), but rarely co-localizes with glial α-synuclein inclusions	**Uryu et al., 2006**
	PD AD	In vivo, human IHC	Astrocytes in all brain areas showed expression of HSPD1/HSP60, which is increased when the cells are reactive.	**Martin et al., 1993**

Table 3: continued

HSP	NDs	Samples and Meth.	HSPs in astrocytes: main findings	Ref.
HSPB (Small Heath Shock Proteins)				
HSPB1 (MT2F; HMN2B; HSP27; HSP28; HSP25; HS.76067; DKFZp586P1322)	AD	In vivo, human IHC	In AD brains, reactive astrocytes were present at the site of neuronal degeneration and amyloid aggregates formation. Reactive astrocytes showed increased expression of HSPB1/HSP27. HSPB1/HSP27 did not co-localize with A-Beta aggregates. ● *See also for HSPB2, HSPB5, HSPB6 and HSPB8 (same study).*	**Bruinsma et al., 2011**
	AD	In vivo, human IHC	In control brains, HSPB1/HSP27 was occasionaly observed in astrocytes. In AD brains, the reactive astrocytes were immunopositive for HSPB1/HSP27. The chaperone occasionaly co-localized with the amyloid plaques (frequency <35%). ● *See also for HSPB5 and HSPB6 (same study).*	**Wilhelmus et al., 2006**.
	AD PD	In vivo, human IHC	In AD brains HSPB1/HSP27 is highly expressed in affected brain regions rich in senile plaques, and notably, in a large number of reactive astrocytes. Expression of HSPB1/HSP27 increased with the severity of AD-specific morphological changes, and with the duration of dementia. Similar patterns of immunoreactivity were present in PD brains although they showed less expression of HSPB1/HSP27 in reactive astrocytes compared to AD brains.	**Renkawek et al., 1994**
	AD (tau)	In vivo, rat model: truncated tau protein into the Thy-1 gene RT qPCR IHC	HSPB1/HSP27 is predominantly overexpressed in reactive astrocytes located in regions with tau pathology, rarely in neurons.	**Filipcik et al., 2015**
	AD	In vivo (mouse model): P301S tau transgenic mice IHC	Reactive astrocytes in the transgenic mice upregulated HSPB1/HSP27. Neurons do not show the same upregulation. Tau aggregates and HSPB1/HSP27 do not co-localize.	**Yata et al., 2011** -

	PD	In vivo, human IHC	HSPB1/HSP27 was upregulated in reactive astrocytes in the brain of PD patients. Such increased expression was correlated with the neuronal degeneration rather with the amount of senile plaques. ● *See also HSPB5 (same study)*	**Renkawek et al., 1999**
	ALS (SOD-1) PD	In vivo, mouse model: - transgene regulated by mouse prion protein promoter. - SOD1G37R for ALS - α-SynA53T for PD IHC	In symptomatic SOD1G37R mice, reactive astrocytes showed increase in HSPB1/HSP25 immunoreactivity in brain regions associated with SOD-1 pathology. Increased HSPB1/HSP25 was not observed in neurons of these regions. Inclusion-like deposit were present in some cells showing increased HSPB1/ HSP25 expression and with astrocyte-like morphology. In symptomatic α-SynA53T mice, similar results are found.	**Wang e al., 2008**
	ALS (SOD-1)	In vivo, mouse model: Leu126delTT mutation in the Cu/Zn superoxide dismutase gene (SOD1) MA RT-qPCR IHC	HSPB1/HSP27 is upregulated in the transgenic SOD-1 mice. HSPB1/HSP27 is markedly increased in post-symptomatic transgenic mice compared to the same animals at disease onset. Moderate increased levels of HSPB1/HSP27 are found in glial cells of transgenic mice at disease onset (compared to a normal control). The expression of HSPB1/HSP27 was localized in the cytoplasm of GFAP-positive glial cells and stronger in reactive astrocytes than microglial cells.	**Fukada et al., 2007**
	ALS (SOD-1)	In vivo,mouse model: human gene for SOD1 with aG93A mutation IHC	Motor neurons of SOD-1 mice showed very little or no immunostaining for HSPB1/HSP25 (whereas the same neurons in control mice showed robust expression of HSPB1/HSP25). In contrast, numerous reactive astrocytes of SOD-1 mice contained HSPB1/HSP25 (Figure 8, second and fourth or bottom panels). Such reactive astrocytes overexpressing HSPB1/HSP25 are diffuse throughout the white matter of all columns. HSPB1/HSP25 and SOD1 co-localized in the cytoplasm of neurons and in some astrocytes. The processes of many reactive astrocytes were immunolabeled with HSPB1/HSP25, but not with SOD1.	**Strey et al., 2004**

Table 3: continued

HSPB2 (MKBP; HSP27; Hs.78846; LOH11CR1K; MGC133245)	AD	In vivo, human IHC	In AD brains, reactive astrocytes were present at the site of neuronal degeneration and amyloid aggregates formation. Reactive astrocytes showed increased expression of HSPB2. HSPB2 co-localized with A-Beta aggregates. ● *See also for HSPB2, HSPB5, HSPB6, and HSPB8 (same study).*	**Bruinsma et al., 2011**
HSPB5 (crystallin alpha B, CRYAB; CRYA2; crystallin)	AD	In vivo, human IHC	In AD brains, reactive astrocytes were present at the site of neuronal degeneration and amyloid aggregates formation. Reactive astrocytes showed increased expression of HSPB5/aB-crystallin. HSPB5/aB-crystallin did not co-localize with A-Beta aggregates. ● *See also for HSPB1, HSPB2, HSPB6 and HSPB8 (same study).*	**Bruinsma et al., 2011**
	AD	In vivo, human IHC	In control brains, HSPB5/aB-crystallin was occasionaly observed in astrocytes. In AD brains, the reactive astrocytes were immunopositive for HSPB4/aB-crystallin. The chaperone did not co-localize with the amyloid plaques. ● *See also for HSPB1 and HSPB6 (same study)*	**Wilhelmus et al., 2006**
	TauoP (i.e.AD)	In vivo, human IHC	Increased expression of HSPB5/alphaB-crystallin in glial cells (including astrocytes) in affected brain regions. Not all glial cells/astrocytes over-expressing HSPB5/alphaB-crystallin were also positive for tau aggregates. Not all astrocytes over-expressing HSPB5/alphaB-crystallin were reactive (increased expression of GFAP)	**Dabir et al., 2004**
	AD	In vivo, human IHC	Increased expression of HSPB5/alphaB-crystallin in reactive astrocytes in AD-affected brain regions	**Tomimoto et al., 1997**
	AD	In vivo, human IHC	Increased expression of HSPB5/alphaB-crystallin in reactive astrocytes in AD-affected brain regions Immunoreactivity to HSPB5/alphaB-crystallin in astrocytes was found mainly restricted to areas with senile plaques and neurofibrillary tangles (tau).	**Renkawek et al., 1994**
	AD	In vivo, human IHC	Increased expression of HSPB5/alphaB-crystallin in astrocytes in AD-affected brain regions	**Shinohara et al., 1993**

	AD ALS PD HD	In vivo, human IHC	Increased expression of HSPB5/alphaB-crystallin in reactive astrocytes in AD-affected brain regions	**Iwaki et al., 1992**
	AD	In vivo, human IHC	Increased expression of HSPB5/alphaB-crystallin in reactive astrocytes in AD-affected brain regions. AD aggregates do not co-localize with HSPB5/alphaB-crystallin.	**Lowe et al., 1992**
	FTLD (TDP-43)	In vivo, human IHC * (*electron microscopy)	TDP-43 aggregates were observed in the brain of the patients and they were were variably positive for the HSPB5/alphaB-crystallin and less often GFAP. Bundles of astrocytic glial fibrils characteristic of reactive astrocytes were often found in proximity, but glial fibrils were negative for TDP-43. These processes might be astrocytic end-feet with abnormal TDP-43 fibrillary inclusions.	**Lin et al., 2009**
	PD (and other α-SynP)	In vivo, human IHC	HSPB5/aB-crystallin is expressed in glial cells (including astrocytes) and often co-localizes with glial α-synuclein inclusions ● *See also for HSPC1 (same study)*	**Uryu et al., 2006**
	PD	In vivo, human IHC	HSPB5/aB-crystallin was upregulated in reactive astrocytes in the brain of PD patients.Such increased expression was correlated with the neuronal degeneration rather with the amount of senile plaques. ● *See also for HSPB1 (same study)*	**Renkawek et al., 1999**
	ALS (SOD-1)	In vivo, mouse model: SOD1-L126Z (Z = stop-truncation of last 28 amino acids) IHC	HSPB5/alphaB-crystallin immunoreactivity was up-regulated in astrocytes of symptomatic mice; Glia cells, including atsrocytes, accumulated mutant SOD1 immunoreactivity.	**Wang et al., 2005**

Table 3: continued

HSPB6 (HSP20; FLJ32389)	AD	In vivo, human IHC	In AD brains, reactive astrocytes were present at the site of neuronal degeneration and amyloid aggregates formation. Reactive astrocytes showed increased expression of HSPB6/HSP20. HSPB6/HSP20 did not co-localize with A-Beta aggregates. ● *See also for HSPB1, HSPB2, HSPB5 and HSPB8 (same study).*	**Bruinsma et al., 2011**
	AD	In vivo, human IHC	In control brains, HSPB6/HSP20 was occasionaly observed in astrocytes in the white and grey matter. In AD brains, the reactive astrocytes surrounding the amyloid plaques were immunopositive for HSPB6/HSP20. ● *See also for HSPB1 and HSPB5 (same study)*	**Wilhelmus et al., 2006**
HSPB8 (AH11; HMN2; CMT2L; DHMN2; E2IG1; HMN2A; HSP22) ***with*** **BAG3** (BCL2 Associated Athanogene 3 ; CAIR1; MFM6)	AD PD HD SCA3	In vivo, human IHC	Strong upregulation of HSPB8 and a moderate upregulation of BAG3 specifically in astrocytes in the cerebral areas affected by neuronal damage and degeneration, for all the investigated diseases. No significant change in the HSPB8-BAG3 expression levels was observed within neurones, irrespective of their localization or of the presence of proteinaceous aggregates.	**Seidel et al., 2012b**
HSPB8 (AH11; HMN2; CMT2L; DHMN2; E2IG1; HMN2A; HSP22)	AD	In vivo, human IHC	In AD brains, reactive astrocytes were present at the site of neuronal degeneration and amyloid aggregates formation. Reactive astrocytes showed increased expression of HSPB8. HSPB8 did not co-localize with A-Beta aggregates. ● *See also for HSPB1, HSPB2, HSPB5 and HSPB6 (same study).*	**Bruinsma et al., 2011**

In table 3, I summarized the available data on responses of astrocytes in terms of HSPs expression as seen in patient brains or in *in vivo* animal disease models. Given the cellular work, these may thus not always and necessarily be due to a direct response of the cells to the presence of aggregation-prone proteins or aggregates. In fact, intra-astrocytic protein aggregates (e.g. tau in AD, α-syn in PD) do not always co-localize with the upregulated HSP in the GFAP-positive astrocytes (studies reported in Table 3). These data suggest that the HSP upregulation in astrocytes might not be exclusively a "stress" response to protein aggregation in that specific cell, but a more concerted intercellular response against the brain damage: astrocytes, in which aggregation is still not initiated, might receive signals (i.e. cytokines) from the neighbour degenerating neurons and overexpress specific protective HSPs in response.

It is, however, striking that astrocytes that show upregulation of HSPs are almost exclusively found in the corresponding region of neurodegeneration where the remaining neurons contain protein aggregates (studies reported in Table 3). In fact, the astrocytes are generally found to up-regulate the specific chaperone at a higher level compared to affected neurons in that area; moreover, also more astrocytes than neurons in a specific brain region are found to be positive to a certain HSP (studies reported in Table 3). On one hand, this might be explained by the neuronal vulnerability to aggregates (neurons degenerate and therefore less cells positive for a certain HSP may be identified); however, this might also substantiate the hypothesis that the HSPs upregulation is part of an astrocytic protective response.

So, the upregulation might fulfil a dual function. It may protect the astrocyte in a cell-autonomous manner, but also protect the neurons in a non-cell autonomous manner. In fact, HSP-boosted astrocytes, which are more resistant to protein aggregation and aggregate toxicity, might keep their functional activity to maintain neuronal fitness and viability. Moreover, the same astrocytes might be capable to release protective HSPs as cargo in exosomes towards neurons or uptake prionoids released by the neurons to impede their spreading in other brain regions (see section 1.2.7.b and Discussion of this Thesis). Nonetheless there is still a general lack of knowledge concerning such aspects.

3.5. Conclusion

The functional implications of the HSPs up-regulation in astrocytes for the progression of neuronal degeneration have not yet been established. The key protective role of the HSPs against protein aggregation and aggregate toxicity suggests that this upregulation might not be merely a stress marker or a compensatory effect, but rather be part of the protective response of astrocytes against diseases characterized by toxic aggregates species.

To get a better insight in the role of astrocytes in HD and whether and how the expression of a specific HSP expression in astrocytes may play a role in this disease, we generated a *D.melanogaster* model of HD that exclusively express the mutant toxic PolyQ HTT in neurons, whilst co-expressing a protective chaperone either in the same neurons or in astrocytes. The chaperone that we choose to investigated is DNAJB6, a member of the human DNAJ family that has potent cell autonomous effects against PolyQ-related neurodegeneration (Hageman et al., 2010; Månsson et al., 2014;

Kakkar et al.,2016) and that has been found to be upregulated in astrocytes in some NDs (Durrenberg et al., 2016).

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CHAPTER 3

A *D.melanogaster* model for the expression of transgenes in neurons and astrocytes

In part based on: Bason M, et al. (2019) Astrocytic expression of the chaperone DNAJB6 results in non-cell autonomous protection in Huntington's disease. Neurobiol Dis. 124:108-117.

ABSTRACT

D. melanogaster is widely used in *in vivo* research because of the availability of flexibile, well-established and -characterised genetic tools that allow the controlled expression of transgenes in a cell-specific or tissue-specific manner, through the use of binary expression systems and specific promoters. Hence, to investigate the role of HSPs expression in neurons and astrocytes in neurodegenerative diseases, we generated a *D.melanogaster* model for the expression of disease-associated polyglutamine (PolyQ) proteins and chaperones. To do so, we used the attP-site specific PhiC31 integrase system of *Drosophila* that allows the site-specific insertion of the transgenic contructs in the fly genome. We employed two different and independent binary expression systems - the GAL4-UAS and LexA-LexO systems - to drive the expression of the PolyQ protein in all neurons and the expression of chaperones in either neuronal cells or in astrocytes. We show that the two expression systems can drive the expression of the transgenes in a completely independent and non-overlapping manner and that levels of expression of the Lex-driven transgene can be controlled by the use of two different LexA trans-activators lines, LG for moderate and LhG for stronger expression. The *D.melanogaster* model generated as such is excellently suited for the investigation of cell-autonomous and non cell-autonomous effects of chaperones in PolyQ pathology and to study the potential interplay between neurons and astrocytes in these diseases.

1. Introduction

The aim of our project was to explore if Heat Shock Proteins (HSPs) - key regulators of the cellular protein quality control (PQC) system (Hartl et al., 2011) – can protect against the toxicity and neurodegeneration mediated by toxic aggregates of PolyQ proteins expressed in neurons, either when such chaperones are expressed in the same neuronal cells (cell-autonomous actions) or when these are expressed exclusively in the astrocytes (non-cell autonomous actions).

Whereas a vast amount of literature shows that specific HSPs in neurons can protect against toxic aggregates associated with neurodegenerative diseases in a cell-autonomous manner (Kakkar et al., 2014), limited to no data are available whether chaperones may also be protective in a non cell-autonomous manner, such as, for example, when they are expressed in non-neuronal cells. Interestingly, however, data from brain of patients and animal models of diseases show that several HSPs of various chaperone families, including small HSP and DNAJ, are up-regulated in astrocytes during neurodegeneration (Chapter 2, section 3.4.2). We hypothesized that such HSP-upregulation in these specific glial cells might not be merely a stress marker or a compensatory effect. Instead, we speculated that this might rather be part of a protective non-cell autonomous response of astrocytes in protein aggregation diseases and that the increased HSP levels in astrocytes might provide an additional level of neuroprotection. The proper evaluation of such a possibility requires the development of an *in vivo* model system allowing the cell-type specific expression of the disease-related proteins and chaperones in either neurons or astrocytes that can be regulated in a tightly controllable, inter-independent manner.

The methodological strategy that we choose was to generate an *in vivo* model for polyglutamine (PolyQ) disease, in which the disease-related protein is driven exclusively in neurons, where it would result in a disease-related phenotype (i.e. reduction in lifespan), caused by the aggregation of the PolyQ protein. To answer our questions, the expression system should allow the co-expression of a protective chaperone in the same neurons, to investigate cell-autonomous protective effects of the chaperone. Next, the model should give the possibility to compare such effects to what happens if the same chaperone is expressed only in astrocytes for eventual non-cell autonomous protective effects. As chaperone, we choose DNAJB6b (Hanai et al., 2003) for which our laboratory had established a comprehensive set of data on its capacity to have strong cell autonomous protective effects in PolyQ diseases, such as Huntington's (Hageman et al., 2010; Kakkar et al., 2016).

D.melanogaster has been considered the ideal choice as *in vivo* model system to conduct our study. The central nervous system of *D. melanogaster* is well characterized in terms of neuronal structures and connections (Rein et al., 2002). Moreveor, several morphologically types of glial cells in the adult *Drosophila* brain have been described, including astrocyte-like cells (Doherty et al., 2009) that are morphologically and molecularly similar to their mammalian counterparts. Astrocytes of *Drosophila* have extended, branched and ramified processes that establish structural and functional contacts with the synapse-rich regions of the fly brain (Doherty et al., 2009). The cell-specific expression of transgenes in the neuronal cells and astrocytes in *Drosophila* is permitted by

the use of well-characterized cell type specific promoters for such cells (Yao et al., 1993; Xiong et al., 1994; Doherty et al., 2009). Controlled and independent expression of the different transgenes is feasible by the use of the binary expression systems of *Drosophila*, i.e. the GAL4-UAS system (Brand and Perrimon, 1993) and the LexA-LexO system (Yagi et al., 2010). In addition, genome-site-specific insertion of the transgene (i.e. the attP-site specific PhiC31 integrase system; Bischof et al. 2007) can be used to avoid "position effects" due to aspecific insertion of the contruct, allowing better comparison of the different phenotypes in the various experimental fly lines (Bischof et al. 2007). Finally, the genetics of *D.melanogaster* is well established and instrumental to the generation and maintenance of the fly lines (Greenspan, 2004).

In this Chapter, the generation of the *D.melanogaster* lines that are used for our experimental studies in chapter 4 and 5 will be described.

2. Materials and methods

Vectors

UAS/LexO vectors were obtained by cloning the sequences of HttQ100-mRFP (Prof. T. Littleton Group, MIT) or V5-DNAJB6 (isoform B) or eGFP (Clontech) in the multiple cloning site of pUAS *attB* or pLexO *attB* (Prof. K. Basler Group, UZH). Driver (Promoter cell-specific expression) vectors were obtained starting from the backbone of plasmids pDPP-Gal4 *attB* or pDPP-LG *attB* or pDPP-LhG *attB* (Prof. K. Basler Group, UZH). DPP promoter was substituted with the sequence of promoter *elav* (pan-neuronal, from p*Elav*-Casper vector, Prof. Liqun Luo, Stanford University), *repo* (pan-glial, from pENTRY-D-TOPO-*Repo*4.3 vector, Prof. C. Klämbt, University of Münster) or *alrm* (astrocytic, from p*Alrm*-Casper vector, Prof. M. Freeman, UMASS). All obtained vectors were sequenced. See table T1 for vectors list.

Genotypes

Fig.4B: 1) active combinations *w(-); UAS eGFP (D) /+; promoter Gal4/+.* 2) inactive combinations: *w(-); UAS eGFP/ promoter LexA; +/+ or w(-); promoter Gal4/+; LexO eGFP (A) /+.*
Fig.5A: *w(-); UAS eGFP/+; promoter Gal4/+ or w(-);promoter LexA (LG or LhG)/+; LexO eGFP/+.* Negative control: *w(-);+/+;+/+.*
Fig.5B: *w(-); UAS eGFP(D)/+; promoter Gal4/+ or w(-);promoter LexA (LG or LhG)/+; LexO eGFP(A)/+.* Negative control: *w(-);+/+;+/+.*

Antibodies and reagents

Antibodies (dilutions are indicated in brackets for western blots (WB) and immunofluorescence (IF)) against huntingtin (Chemicon, MAB2166, WB 1:5000), eGFP (Clontech-Living Colours, cat.No.632375, WB 1:5000), α-tubulin (Sigma Aldrich, clone DM1A, WB 1:2000), V5 epitope tag in DNAJB6b (Thermo Fisher Scientific, cat. No.R960-25, WB 1:2000, IF 1:50), NC-82 (DSHB, WB 1:5000) were used. DAPI for nuclei staining (cat.No.D1306) was from Thermo Fisher Scientific. 20% SDS Solution (cat.No.1610418) was from BioRad. PBS components (NaCl cat.No.S9888, KCl cat.No.P9541, Na2HPO4 cat.No.255793, KH2PO4 cat.No.V000225), Tween-20 (cat.No.P2287), Triton X-100 (cat.No.T8787), Bovine Serum Albumin (cat.No.A2058, BSA), glycerol (cat.No.G5516), 3.7% Formaldehyde (cat.No.11-0705 SAJ), Tris base (cat.No.T1503) and β-mercaptoethanol (cat.No.M6250) were from Sigma Aldrich.

D. melanogaster stocks maintenance

All stocks and experimental flies were kept in polystyrene vials 25x95 mm filled with 8 ml/vial of solidified media (17 g/l Agar; 26 g/l Yeast; 54 g/l Sugar; 1.3 mg/l Nipagin). All experimental flies were maintained in a humidified and temperature controlled incubator at 25 °C on a 12 hours' light and 12 hours' dark cycle (Premium ICH Insect Chamber, Snijders Labs). Experimental flies, anesthetized on a CO2 pad, were selected according to their gender and phenotype by light microscope visualization.

Western Blotting *D. melanogaster* total head lysates preparation

30-40 *D. melanogaster* adults with specific phenotype, gender, age (days after pupal eclosion) and condition were collected; after freezing in liquid nitrogen and vortexing of entire flies, separated heads were collected, counted and lysed in SDS-rich buffer (SDS 1.45%, Glycerol 20%; Tris Base 0.2 M. 2.5 µl of buffer/head) using sonication (3 pulses of 50 Watt for 5 seconds). Homogenized lysate was then centrifuged at 1000 x g for 3 seconds to separate cuticle debris from supernatant. Proteins in supernatant were collected and quantified using spectrophotometry (Implant NanoPhotometer UV/Vis). Protein content was equalized. Samples, supplied with β-mercaptoethanol 5% and bromophenol blue, were boiled at 99 °C for 5 minutes. Equal amounts of volume were resolved on SDS-PAGE. Flies of the same line were collected from different vials and the entire experiment was repeated at least 2 times.

Western Blotting and Blot quantification

Following the preparation of samples, proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and processed for Western Blotting. Primary antibodies (at concentrations mentioned above) were prepared in 3% BSA/PBS-Tween 20 0.1%, secondary antibodies at concentration 1:5000 (Invitrogen, horse peroxidase conjugated to IGG or IGM) in 5% milk/PBS-Tween 20 0.1%. For visualization membranes were incubated with Pierce ECL Western Blotting

substrate (cat. No. 32106) for 2 minutes and visualized using ChemiDoc Touch Imaging System (BioRad). Blots have been quantified using Image Lab Version 5.2.1 software (BioRad).

3. Results and Discussion

To generate the *D.melanogaster* model for comparing cell-autonomous and non-cell autonomous effects of chaperones in neurodegenerative diseases, we used the GAL4-UAS (Brand and Perrimon, 1993) and LexA-LexO (Yagi et al., 2010) expression systems of *Drosophila*. These systems have been shown to drive expression of transgenes in a completely independent and non-overlapping manner.

The expression of two different transgenes in specific cell types (i.e. neurons or astrocytes) of *D.melanogaster* brain is allowed by the use of cell-specific and well-characterized promoters ("drivers") in the expression system. For the pan-neuronal expression of transgene, we used the promoter *elav* (Yao et al., 1993). To selectively express the second transgene in astrocytes, we used the promoter *alrm* (Doherty et al., 2009). Alternatively to *alrm,* and as additional control, we used the pan-glial promoter *repo* (Xiong et al., 1994) to express the second transgene in all glial cells.

The disease-causing PolyQ protein used in our *D.melanogaster* model of neurodegenerative disease is Huntingtin (HTT). We used a construct, hereafter referred to as HttQ100-mRFP, encoding for human PolyQ-HTT exons 1-12 (Weiss et al., 2012). As previously said, the chaperone used in our study is human DNAJB6 (short nuclear and cytosolic isoform B; Hanai et al., 2003).

3.1 Generation of the D. melanogaster lines

To generate the *D.melanogaster* lines for our experiments, the sequences of the above-indicated cell-specific promoters or the transgenes (for the PolyQ protein and the chaperone) were cloned into the respective GAL4-UAS and LexA-LexO plasmids, and sent to Best Gene Inc. injection service (https://www.thebestgene.com/HomePage.do), for injection and transformation of embryos of *Drosophila* (see Material and Methods and table T1).

These plasmids carry an *attB* site that allows the site specific insertion of the contructs in the fly genome, through the use of the FlyC31 system (Bischof et al. 2007). In this system, established by the Basler group, a library of several *D. melanogaster* lines have been generated, each line containing a specific landing platform with an attP site. Several lines of FlyC31 system are available with different positions of the attP landing site in the *D. melanogaster* genome (including chromosomes 2 and 3). The generation of the transgenic flies is based on the use of PhiC31 integrase, a serine-type recombinase that mediates the sequence-specific recombination between two largely different attachment sites, *attB* and *attP*, which share a 3 bp central region where such crossover occurs (Thorpe et al., 2000). This allows the transgenesis of the *attB* construct at the attP landing site.

Differently from other systems to generate transgenic flies (such as the P-element mediated transformation), the integration of the *attB* vector at the *attP*-site in the fly genome using the PhiC31 integrase is really efficient, irreversible and, more importantly, site-specific at the desired location (non-random integration). First, this allows to pre-select the positions of the constructs on the fly chromosomes, allowing to plan the required crosses to obtain the flies with the desired genotypes for the experiments in advance. Second, since the position of the transgenic sequence is controlled, all constructs integrated into the same locus can be directly compared (i.e. eGFP-expressing construct as control, and DNAJB6-expressing construct as experimental condition). Third, the *attP* landing sites are selected to avoid insertion of the construct into life-essential endogenous fly genes or nearby enhancers and silencers sequences which can affect the transgene expression ("position effect") in an unpredictable manner (Bischof et al. 2007).

Transgenic flies were detected by verifying the presence of the "red eyes" marker (w+) and an RFP marker that is expressed in the eyes of the adult via the 3xP3 promoter. The genetic sequences for these two markers are located at the site of insertion of the construct and are between LoxP sequences (Bischof et al. 2007). When needed (e.g. in experiments using fluorescence signals from the transgenes), the sequences for these two markers were removed by using a CRE-Recombinase line. The removal of the two markers were verified at the optical and fluorescent microscopes respectively. Importantly, the remaining transgenic sequences of the construct (e.g. the sequence for UAS-transgene of interest) are not removed by the CRE-recombinase (Bischof et al. 2007). All the lines were isogenised to remove background mutations by backcrossing each of them for 6 generations with the control stock w^{1118} line.

3.2 Binary expression and crossing

All the crossings required to generate the experimental flies described below were performed following the instructions and recommendations of the manual "Fly Pushing - The Theory and Practice of Drosophila Genetics" by Ralph J. Greenspan. For crossings, lines carrying balancer chromosomes, such as IF/CyO (for the second chromosome) or MKRS/TM6B (for the third chromosome) were used. Balancer chromosomes carry specific dominant markers which can be easily identified from the phenotype of the flies (e.g. flies carrying the marker CyO show curly wings), allowing the proper selection of desired flies for crossing or experiments. The use of such balancers prevents recombination between homologous chromosomes during the meiosis in the selected flies.

As an example for use of these balancer lines in *Drosophila*, flies carrying the transgene A, (w+) / + (red eyes with normal shape, normal wings) at the second chromosome can be first crossed with the balancer line IF/CyO (white eyes with abnormal shape, curly wings) to obtain the heterozygous line A/CyO which is easily recognizable by having red normal eyes and curly wings (Greenspan, 2004). The offspring of these A/CyO flies can be still A/CyO or A/A (red eyes, normal wings) (Greenspan, 2004), allowing the maintenance of a population of flies respectively carrying the heterozygous or homozygous transgene. The line CyO/CyO is not viable (Greenspan, 2004). Similarly, flies carrying a transgene B at the third chromosome can be crossed with a balancer line

such as MKRS/TM6B (Greenspan, 2004), to obtain the needed offspring B/TM6B for further crossing and experiments.

An expression system such as GAL4-UAS is constituted by two constructs (Fig.1): the first carries the cell-specific promoter ("driver") that drives the expression of a transcriptional activator (the "trans-activator"), like the yeast-derived GAL4 protein; the second carries the sequence of the desired transgene, which is under the control of a promoter with an enhancer sequence, like the upstream activation sequence (UAS), that is specific to and recognized by the GAL4 protein. Importantly GAL4 (and trans-activators of other expression systems) lacks endogenous targets in *D.melanogaster.* The fly with the construct with the driver-GAL4 can next be crossed with another line with the construct UAS-gene of interest. In the offspring lines carrying both the constructs, the GAL4 protein will now bind the UAS sequence and activate the gene expression in the cells that the driver is specific for. Besides the possibility to study the expression of the desired gene in specific cells by using the various cell-specific promoters available, one of the advantages of this system is also that the transgenes are not expressed in the parental lines, but only in the offspring (first generation) obtained after the crossing. In this way, the parental lines carrying a toxic gene (e.g. a line with UAS-PolyQ-HTT) do not express it and can survive normally.

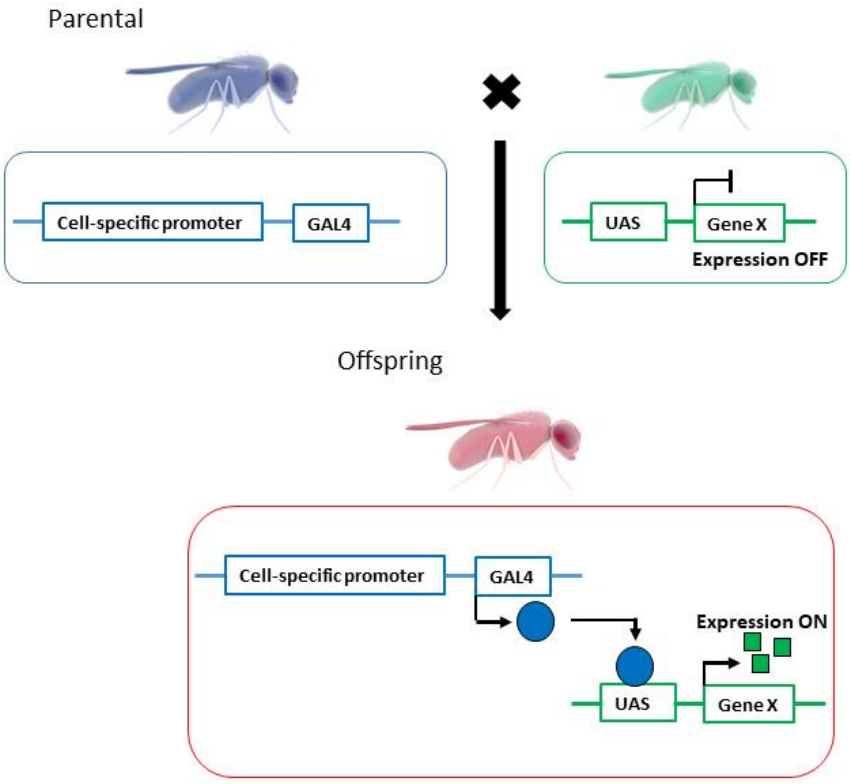

Figure 1: the GAL4-UAS system. *See main text for explanation.*

In our project, we required a second, independent system for which we selected the LexA-LexO binary expression system (Fig.2). This system is based on the use of a trans-activator named LexA - a bacterial transcription factor - that binds specific sequences called LexO (LexA Operator). Similar to the GAL4-UAS system, here the cell-specific promoter drives the expression of LexA that can bind LexO in the promoter of the transgene of interest, allowing its expression. For our investigation, we made use of a refined LexA-LexO that was designed to be used independently in combination with the GAL4-UAS system (Brand and Perrimon, 1993). In additions, for this system two different LexA trans-activators are available: LG for moderate expression and LhG for stronger expression. In the latter, the transcriptional activity was improved by introducing the hinge region (H) of GAL4 in the original sequence of LG (Yagi et al., 2010).

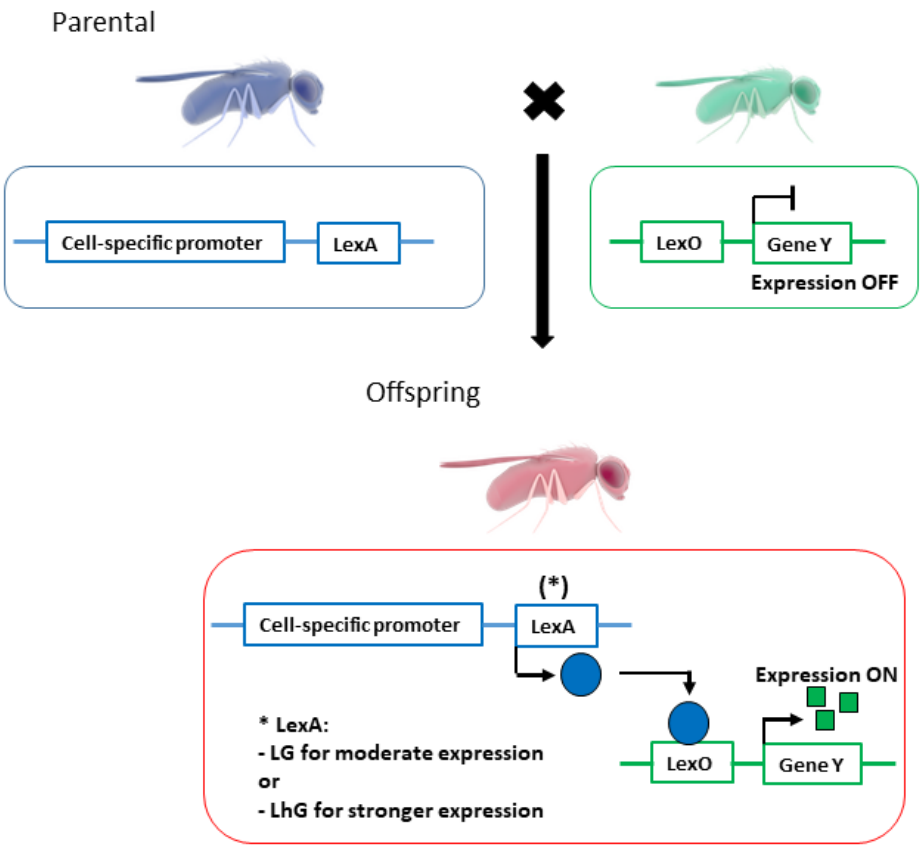

Figure 2: the LexA-LexO system. *See main text for explanation.*

As previously said, in our investigation, we used GAL4-UAS and LexA-LexO in combination to express two transgenes in different cell types in the same fly. The combined use of the two systems to co-express two transgenes in different cell types in the same fly is illustrated in Fig.3. By making use of the balancer lines with a distinct phenotype (see above), we first obtained the needed parental flies. By crossing the first parental line carrying the two drivers constructs (e.g. *elav*-GAL4 and *alrm*-LexA) with the second parental line carring the two transgene constructs (e.g. UAS-eGFP and LexO-RFP), we obtained an offspring in which each transgene is driven by its respective cell-specific promoter (i.e. eGFP in neurons and RFP in astrocytes of the *Drosophila* brain) (Fig. 3).

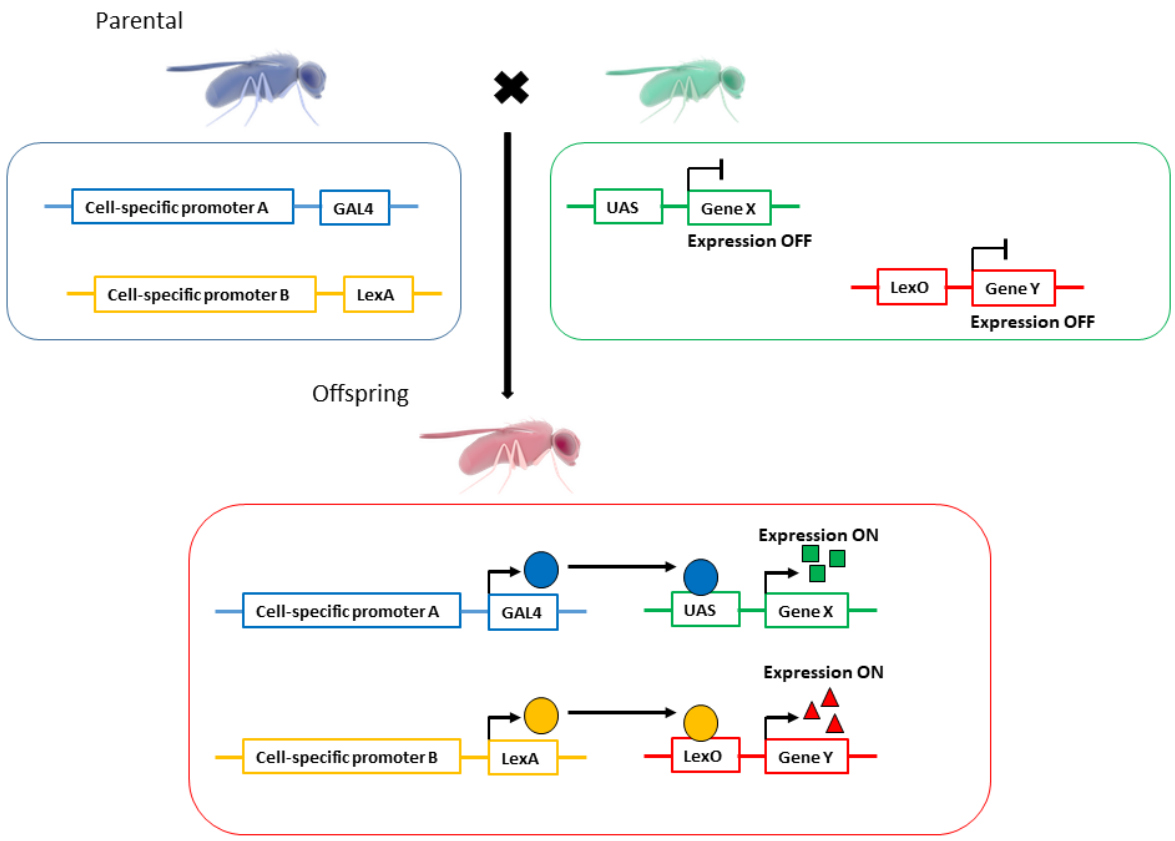

Figure 3: schematic of crossing and use of the GAL4-UAS and LexA-LexO systems in combination, to co-express two transgenes in different cell types. *See main text for explanation.*

3.3. Verification of the expression systems

To indeed verify that the two expression systems can drive the expression of the transgenes and in a completely independent and non-overlapping manner in the generated *Drosophila* lines, we monitored the expression of the fluorescent model protein eGFP expression as highly sensitive readout. To do so, we crossed the parental lines to obtain an offspring carrying a combination of constructs which can be positive or negative for the expression of eGFP. Whereas positive combinations (i.e. *promoter*-GAL4 > UAS eGFP or *promoter*-LexA > LexO eGFP) should lead to expression of eGFP, a negative combination (i.e. *promoter*-GAL4 > LexO eGFP or *promoter*-LexA > GAL4 eGFP) should not (Fig.4A).

Next, we verified in the experimental flies, by western blot analyses, that the inactive combinations (i.e. GAL4-LexO or LexA-UAS), in contrast with the active one (GAL4-UAS), do not lead to eGFP expression in the generated *D.melanogaster* lines (Fig.4B). Importantly, we verified this for the neuronal (*elav*), astrocytic (*alrm*) and pan-glial (*repo*) promoters, both in male and female flies (Fig.4B). These data indicate that the two sytems are independent each other, implying that our *Drosophila* system can be used as a tool to investigate the interplay between neurons and astrocytes *in vivo*. In effect, these results indicate that each transgene is expressed in the desired cell types, therefore allowing the investigation of mechanisms such as the intercellular prion-like spreading of

the disease-causing protein (section 2.6 of Chapter 2) or the release of chaperones via vesicle release from one cell type to another (Takeuchi et al., 2015), excluding that this might instead due to a leakage of the expression systems.

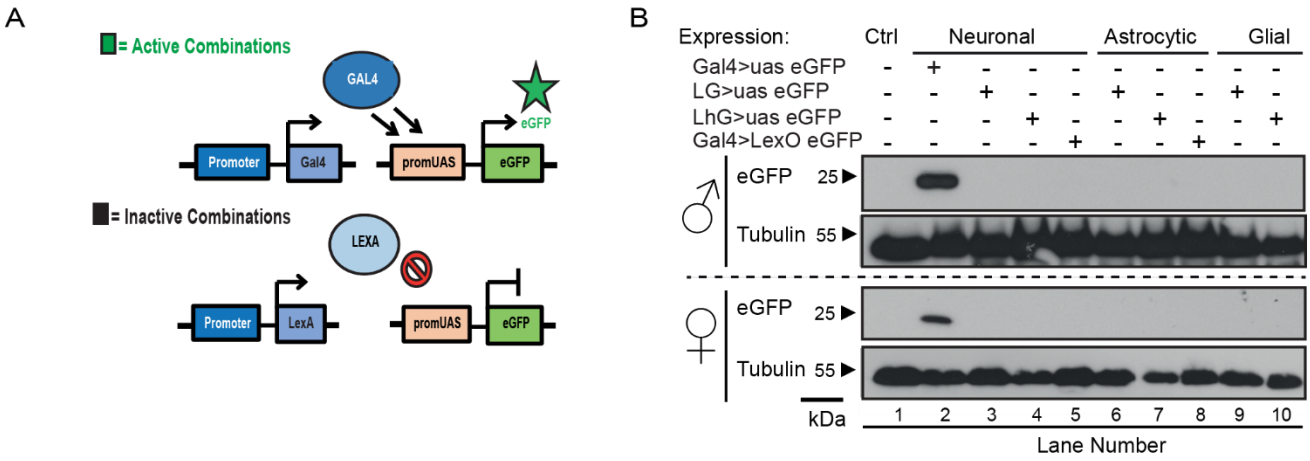

Figure 4: Validation of the Gal4-UAS and LexA-LexO expression systems A) *Schematic explanation and experimental confirmation (by using eGFP expression) of the independent Gal4-UAS and LexA-LexO expression systems. In the D. melanogaster lines carrying an "active combination" of a binary expression system, a cell-specific promoter (i.e. neuronal-, glial- or astrocytic- specific) drives the expression of the trans-activator (GAL4 or LexA) that recognizes and binds a specific sequence in the promoter of eGFP (UAS or LexO respectively), leading to the final expression of the transgene. In the "inactive combination", the trans-activator does not recognize such sequence (i.e. in a line in which the trans-activator LexA is combined with the UAS sequence).* ***B)*** *Western blots of total head lysates of 15-day-old adult male and female flies in which the transgene UAS-eGFP or LexO-eGFP is driven (or not) by the Gal4 or LexA (LG or LhG) promoters (neuronal: elav, lanes 2-5; astrocytic: alrm, lanes 6-8; glial: repo, lanes 9-10). Active and inactive combinations, as defined in Fig.4A, are shown. Anti-GFP antibody for eGFP detection. Tubulin was used as loading control. Number of fly heads per each lysate sample and genotypes in Materials and Methods.*

We also investigated the two different LexA trans-activators lines, LG for moderate and LhG for stronger expression. In Fig.5A-B, the levels of different eGFP expression, using moderate LG promoters or stronger LhG promoters (for neuronal, glial or astrocytic expression), are shown and compared with the GAL4-UAS mediated expression. Again, we verified this for the neuronal (*elav*), astrocytic (*alrm*) and pan-glial (*repo*) promoters, both in male and female flies (Fig.5A and 5B). The blots confirm that LhG promoters are stronger than the LG promoters, as also previously reported (Yagi et al., 2010). The possibility of titrating the level of expression of the transgene under the control of the LexA-LexO system opens to the possibility to compare how such level can influence the experimental read-outs. For instance, in our research the chaperone is under the control of the LexA-LexO system. By using the LG or the LhG drivers, we can investigate and compare how the level of expression of the HSP in neurons or astrocytes affects the phenotype of the Huntington's model, exploring cell-autonomous and non-cell autonomous mechanisms. Chaperones can affect the aggregation and therefore toxicity of disease-causing PolyQ proteins and *in vitro* experiments indicate that the level of the HSPs is a key factor for protection and anti-aggregation activity (Månsson et al., 2014). Our model represents an advance because it allows to explore such aspects in an *in vivo* context.

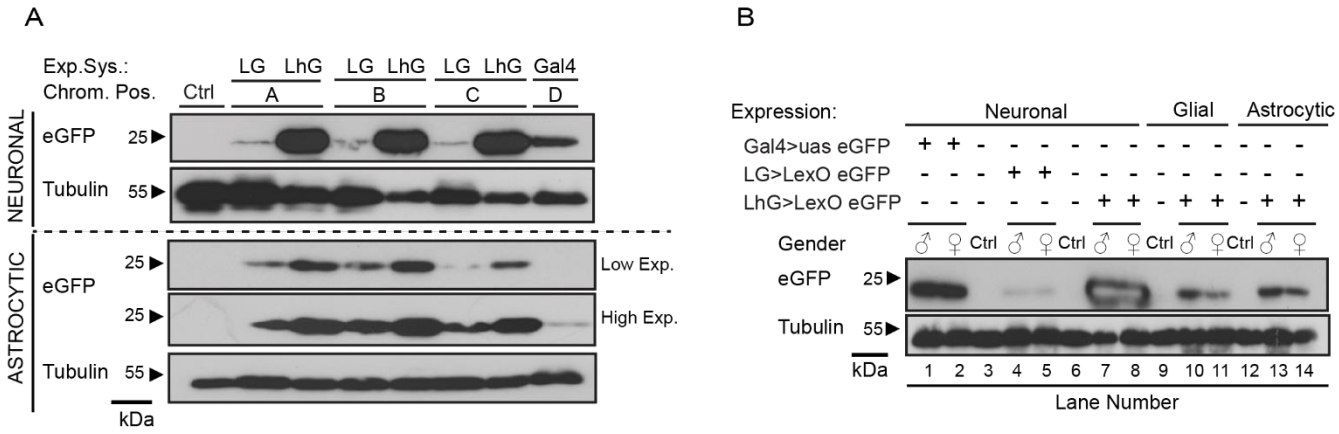

Fig.5: Level of transgene expression using the GAL4-UAS or LexA-LexO expression systems. A) *Western blots of total head lysates of 15-day-old male adult flies in which the transgene UAS-eGFP or LexO-eGFP is driven by the GAL4 or LexA promoter (LG or LhG) in neurons (elav) or astrocytes (alrm). eGFP lines with different chromosome insertion positions (Table T1) are shown. Anti-GFP antibody for eGFP detection. Tubulin was used as loading control. Number of fly heads per each lysate sample and genotypes in Materials and Methods.* ***B)*** *Western blots of total head lysates of 15-day-old adult male and female flies in which the transgene UAS-eGFP or LexO-eGFP is driven by the GAL4 or LexA (LG or LhG) promoters (neuronal: elav, lanes 1-8; glial: repo, lanes 9-11; astrocytic: alrm, lanes 12-14). Levels of eGFP expression in different genders and promoters are shown. Anti-GFP antibody for eGFP detection. Tubulin was used as loading control. Number of fly heads per each lysate sample and genotypes in Materials and Methods.*

In conclusion, we generated a *D.melanogaster* model that allows the independent and non-overlapping expression of two different transgenes of interest in different population of cells - neurons and astrocytes (or all glial cells) - of the fly brain, by using different cell-specific promoters and the GAL4-UAS and LexA-LexO systems. The use of the *attP*-site specific PhiC31 integrase system (Bischof et al. 2007) to generate these lines avoids "position effects" due to random integration of the transgenes in the fly genome. Moreover, such pre-determined insertion of the contructs in equivalent positions into the genome consents high comparability between the different lines. Finally, the use of two different trans-activators LG or LhG - also allows to titrate the level of expression of the transgene under the control of the LexA-LexO system (Yagi et al., 2010). All together, the data above indicate that the generated *Drosophila* model is ideal to answer our research questions and open to intriguing further perspectives.

3.4 Perspectives

The transgenic expression in neurons or astrocytes opens to the possibility to investigate the *in vivo* effects of such genetic manipulation when the same gene is expressed in the two different cell types (e.g. the reduction in lifespan of a toxic PolyQ protein when expressed in neurons or glial cells). More importantly, two different transgenes can be expressed at the same time in the two cell populations and this allows to investigate both cell-autonomous and non-cell autonomous effects.

Our model can find use in investigating the role of chaperones in the brain, against the toxicity mediated by the expression of disease-causing proteins associated with neurodegenerative diseases

(as it will be illustrated in the Chapters 4 and 5). As previously explained, the protective chaperone DNAJB6 (Hageman et al., 2010; Kakkar et al., 2016) can be co-expressed with PolyQ Huntingtin in the same neurons. In a parallel experiment, using a different set of driver lines, the same chaperone DNAJB6 can be expressed in astrocytes whereas PolyQ Huntingtin is expressed in neurons. Hence, the non-cell autonomous effects and interplay between neurons and astrocytes can be explored and comparison with the effects observed in other combinations can be made. Nonetheless, the model can be used to explore other mechanisms and pathways between neurons and astrocytes, not only by over-expression of transgenes but also by the cell-specific silencing of certain genes.

In conclusion, our new established model highlights the potential of *D.melanogaster* as *in vivo* research tool to investigate the complex interlinks between neurons and astrocytes and paves the way to explore possible therapeutic options that exploit the crucial role of astrocytes in maintaining the neuronal fitness and viability in normal conditions, but also during disease (Chapter 2).

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SUPPLEMENTARY DATA

Line Name	Plasmid	*D.melanogaster* Genotype	Chromosome	Chromosome Arm	PhiC31 *attP* -Site
Driver (Cell-Specific) Lines					
elav-Gal4	p Elav-Gal4 *attB*	*w(-); +/+; elav-Gal4*	3	R	ZH-attP-86Fb
alrm-Gal4	p Alrm-Gal4 *attB*	*w(-); +/+; alrm-Gal4*	3	R	ZH-attP-96E
elav-LG	p Elav-LG *attB*	*w(-); elav-LG; +/+*	2	L	ZH-attP-22A
elav-LhG	p Elav-LhG *attB*	*w(-); elav-LHG; +/+*	2	L	ZH-attP-22A
repo-LG	p Repo-LG *attB*	*w(-); repo-LG; +/+*	2	L	ZH-attP-35B
repo-LhG	p Repo-LhG *attB*	*w(-); repo-LHG; +/+*	2	L	ZH-attP-35B
alrm-LG	p Alrm-LG *attB*	*w(-); alrm-LG; +/+*	2	L	ZH-attP-35B
alrm-LhG	p Alrm-LhG *attB*	*w(-); alrm-LHG; +/+*	2	L	ZH-attP-35B
UAS/LexO-TRANSGENE Lines					
UAS HttQ100-mRFP	p UAS HttQ100-mRFP attB	*w(-); UAS HttQ100-mRFP; +/+*	2	R	ZH-attP-51C
UAS DNAJB6	p UAS V5-DNAJB6 attB	*w(-); UAS V5-DNAJB6; +/+*	2	R	ZH-attP-58A3
UAS eGFP (D)	p UAS eGFP attB	*w(-); UAS eGFP; +/+*	2	R	ZH-attP-58A3
LexO V5-DNAJB6	p LexO V5-DNAJB6 *attB*	w(-);+/+; LexO V5-DNAJB6	3	L	ZH-attP-68E1
LexO eGFP **(A)**	p LexO eGFP *attB*	w(-);+/+; LexO eGFP	3	L	ZH-attP-68E1
LexO eGFP **(B)**	p LexO eGFP *attB*	w(-);+/+; LexO eGFP	3	L	ZH-attP-62B
LexO eGFP **(C)**	p LexO eGFP *attB*	w(-);+/+; LexO eGFP	3	L	ZH-attP-75D

Table T1. D.melanogaster lines generated in this study. T1) *D.melanogaster lines generated by injection and transformation of embryos with attB plasmids, based on attP-site specific PhiC31 integrase system, by Best Gene Inc.*

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CHAPTER 4

Neuronal expression of the chaperone DNAJB6 results in cell autonomous protection in Huntington's disease

In part based on: Bason M, et al. (2019) Astrocytic expression of the chaperone DNAJB6 results in non-cell autonomous protection in Huntington's disease. Neurobiol Dis. 124:108-117.

ABSTRACT

Neurodegenerative diseases (NDs) like Huntington's (HD) and Spinocerebellar Ataxia type 3 (SCA-3) are characterized by protein aggregation in brain cells, due to the abnormal elongation of a Polyglutamine (PolyQ) repeat in the mutated protein. Aggregation of PolyQ huntingtin (HTT) or PolyQ ataxin-3 (ATXN3) is believed to be the main driver of pathology and degeneration. Heat Shock Proteins (HSPs) are regulators of the protein quality control system and are thought to be protective against the aggregation of disease-related proteins and thereby capable to delay the onset of these NDs. DNAJB6 is a human HSP of the DNAJ family and HSP70 co-chaperone that has been identified as possessing potent anti-PolyQ aggregation properties in different *in vitro* and *in vivo* PolyQ disease models. Here, we demonstrate that the protective cell autonomous function of DNAJB6 is conserved in a *D. melanogaster* ommatidia models of both HD and SCA3, and that this activity is generic for the core PolyQ-expansion of the protein. Next, we show that the selective neuronal expression of human DNAJB6 expands lifespan, reduces aggregate formation and improves overall neuronal fitness in a pan-neuronal PolyQ Htt *D. melanogaster* model, supporting that DNAJB6 is a potent suppressors of PolyQ aggregation within the human chaperonome and providing further evidence for the cell-autonomous protective role of this DNAJ in PolyQ diseases.

KEYWORDS

Neurodegeneration - Polyglutamine - Aggregation - Neurons - Chaperones - DNAJB6

1. Introduction

Huntington's disease (HD, OMIM:#143100) and spinocerebellar ataxia type 3 (SCA-3, OMIM:#109150) are neurodegenerative diseases characterized by an abnormal trinucleotide CAG-expansion in a specific gene. The encoded mutant protein - huntingtin (Htt) in HD and ataxin-3 (ATXN3) in SCA-3 - contains an elongated polyglutamine (PolyQ) stretch and forms aggregates that are considered to be the main driver of neuronal degeneration and pathology. The length of the PolyQ expansion is linked with the propensity of the protein to aggregate and it is inversely correlated to the age of onset, suggesting that aggregate formation is the main driver of disease (Chapter 2, section 2.3).

PolyQ aggregates can exert their toxicity through different mechanisms, which mainly depend on the cellular localization of these toxic species and their capacity to interact and interfere with the normal functionality of different cellular components, including proteins and organelles. All together such mechanisms contribute to the functional impairment of neurons and ultimately to their degeneration. Damage of specific population of neurons in HD (i.e. medium spiny neurons in striatum and cortical neurons) and SCA-3 (i.e. neurons in brainstem and cerebellum) corresponds to the insurgence of specific symptoms in patients, including cognitive impairment and motor disabilities (i.e. chorea in HD) (Chapter 2, section 2.3 - 2.4).

Molecular chaperones, also known as Heat Shock Proteins (HSPs), play a key role in protein homeostasis (Hartl et al., 2011) and they are known to exert a protective cell-autonomous function against aggregate toxicity, hence neurodegenerative diseases characterized by protein aggregation (Hageman et al., 2011; Vos et al., 2010; Hageman et al., 2010; Kakkar et al., 2016b).

Several chaperones - defined here as canonical HSP - are up-regulated upon stress (i.e. including conditions which are known to drive protein aggregation) during a process referred as heat shock response (HSR), which is controlled by the transcription factor heat shock transcription factor-1 (HSF-1). In animal model of PolyQ diseases, the activaction of HSR (Labbadia et al., 2011) or the transgenic overexpression of specific canonical HSP such as HSPA1A/HSP70 (Hansson et al., 2003; Hay et al., 2004) and HSPB1/HSP27 (Zourlidou et al., 2007) have generally not been found to delay disease onset or reduce aggregate formation. Also, the expression of PolyQ proteins does not activate the HSR (Seidel et al., 2012). These findings suggest that canonical HSP are ineffective in PolyQ diseases.

In a screen for PolyQ modifiers, our group identified two non-canonical HSP, close homologs of the DNAJ/HSP40 chaperone family, called DNAJB6 (expressed in several tissues, including the brain) and DNAJB8 (expressed in testis), that are not significantly activacted by HSF-1 (Hageman et al., 2010). We found that DNAJB6 (the short nuclear and cytosolic isoform B; Hanai et al., 2003) is a very efficient suppressors of PolyQ aggregation in free-cell, cellular and animal models (Hageman et al., 2010, Gillis et al., 2013, Mansson et al., 2014, Kakkar et al., 2016). Notably, the suppression of PolyQ aggregation mediated by DNAJB6 is protective in cells (Hageman et al., 2010) and delays disease onset and significantly prolong lifespan in a mice model of PolyQ disease (Kakkar et al., 2016).

Here, we investigated whether the cell-autonomous protective function of human DNAJB6 against PolyQ aggregation was also maintained in *D. melanogaster* models of HD and SCA-3 (Chapter 3 and this Chapter). By using *D. melanogaster*, we confirmed and recapitulated the previous findings that DNAJB6 is protective against PolyQ aggregate toxicity *in vivo*, through the suppression of PolyQ Htt aggregation, resulting in a significant expansion of the lifespan and improved neuronal fitness *in vivo*. Moreover, these findings setted the base to further investigate the non-cell autonomous protective function of DNAJB6 (Chapter 5). As previously explained, the use of the *D.melanogaster* model generated with the attP-site specific PhiC31 integrase system, allowed to compare how differently DNAJB6 can exert a protective function against Poly Htt toxicity in a cell-autonomous (data in this Chapter) or non-cell autonomous (data in Chapter 5) manner.

2. Materials and methods

Vectors

UAS/LexO vectors were obtained by cloning the sequences of HttQ100-mRFP (Prof. T. Littleton Group, MIT) or V5-DNAJB6 (isoform B) or eGFP (Clontech) in the multiple cloning site of pUAS *attB* or pLexO *attB* (Prof. K. Basler Group, UZH). Driver (Promoter cell-specific expression) vectors were obtained starting from the backbone of plasmids pDPP-Gal4 *attB* or pDPP-LG *attB* or pDPP-LhG *attB* (Prof. K. Basler Group, UZH). DPP promoter was substituted with the sequence of promoter *elav* (pan-neuronal, from p*Elav*-Casper vector, Prof. Liqun Luo, Stanford University). All obtained vectors were sequenced. See table T1 of Chapter 3 for vectors list.

Generation of new *D. melanogaster* lines

The *D. melanogaster* lines of table T1 were obtained by injection and transformation of embryos with the above mentioned *attB* vectors, based on *attP*-site specific PhiC31 integrase system, by Best Gene Inc. injection service (https://www.thebestgene.com/HomePage.do). *D. melanogaster* lines from Bloomington Drosophila Stock Center were also used: *gmr-Gal4* (Line BDSC #8121 in Fig.1); *gmr-Gal4* (Line BDSC #1104 in Fig.2); *alrm-Gal4* (Line BDSC #67031 in Fig.2); UAS-mCD8-GFP (Line BDSC #5130); UAS-mCD8-RFP (Line BDSC #27391); UAS-ATXN3-Q78 (Line BDSC #8150 in Fig.2); *gmr-QF2* (Line BDSC #59283 in Fig.2 was a gift from C. Potter, Baltimore, MD, U.S.A.). *gmr-QF2* and QUAS-ATXN3-Q78 are based on the Q expression system in *D.melanogaster* (Potter et al., 2010). All the lines were isogenised to remove background mutations by backcrossing each of them for 6 generations with the control stock w^{1118} line. See Chapter 3 for other details.

Genotypes

- Fig. 1: *w(-); UAS HttQ100-mRFP / + (or UAS DNAJB6); gmr Gal4 / UAS mCD8-GFP (or +)*.
- Fig. 2: *w(-); gmr-Gal4:UAS ATXN3-Q78/ UAS DNAJB6 (or +); +/+.*
- Fig.3A, S1A: *w(-); UAS HttQ100-mRFP (or UAS eGFP) / +; elav Gal4/+.*
- Fig 4B and S2D: 1) Control line (red): *w(-); UAS HttQ100-mRFP / elav LhG; elav Gal4 / LexO eGFP.* 2) Rescued line (blue): *w(-); UAS HttQ100-mRFP / elav LhG; elav Gal4 / LexO DNAJB6.*
- Fig. 5A-B and S4C-D: 1) Control line (red): *w(-); UAS HttQ100-mRFP / elav LhG; elav Gal4 / LexO eGFP.* 2) Rescued line (blue): *w(-); UAS HttQ100-mRFP / elav LhG; elav Gal4 / LexO DNAJB6.*
- Fig.S4A-B: *w(-); UAS HttQ100-mRFP (or +) / +; elav Gal4/+.*

Antibodies and reagents

Antibodies (dilutions are indicated in brackets for western blots (WB) and immunofluorescence (IF)) against huntingtin (Chemicon, MAB2166, WB 1:5000), eGFP (Clontech-Living Colours, cat.No.632375, WB 1:5000), α-tubulin (Sigma Aldrich, clone DM1A, WB 1:2000), V5 epitope tag in DNAJB6b (Thermo Fisher Scientific, cat. No.R960-25, WB 1:2000, IF 1:50), NC-82 (DSHB, WB 1:5000) were used. DAPI for nuclei staining (cat.No.D1306) was from Thermo Fisher Scientific. 20% SDS Solution (cat.No.1610418) was from BioRad. PBS components (NaCl cat.No.S9888, KCl cat.No.P9541, Na2HPO4 cat.No.255793, KH2PO4 cat.No.V000225), Tween-20 (cat.No.P2287), Triton X-100 (cat.No.T8787), Bovine Serum Albumin (cat.No.A2058, BSA), glycerol (cat.No.G5516), 3.7% Formaldehyde (cat.No.11-0705 SAJ), Tris base (cat.No.T1503) and β-mercaptoethanol (cat.No.M6250) were from Sigma Aldrich.

D. melanogaster stocks maintenance

All stocks and experimental flies were kept in polystyrene vials 25x95 mm filled with 8 ml/vial of solidified media (17 g/l Agar; 26 g/l Yeast; 54 g/l Sugar; 1.3 mg/l Nipagin). All experimental flies were maintained in a humidified and temperature controlled incubator at 25 °C on a 12 hours' light and 12 hours' dark cycle (Premium ICH Insect Chamber, Snijders Labs). Experimental flies, anesthetized on a CO2 pad, were selected according to their gender and phenotype by light microscope visualization.

Lifespan curves

Parental flies (5-6 females and 5-6 males) were kept in vial for 3 days and then removed. Offspring virgin flies were collected in the same 24 hours. For each analysed group, ≈100 flies of specific gender and phenotype were collected and kept in new vials (10 flies/vial). Flies were transferred to new vials containing fresh medium every 2 days and deaths were scored daily. Statistical significance of curves differences analysed with Log rank (Mantel-Cox) test (test 1) and Gehan-Breslow-Wilcoxon test (test 2) using Graph Pad Prism Software Version 5.00. All curves comparisons were made from

flies analysed in the same experiment. T50 was defined as the time point at which 50% of the initial population has died.

Western Blotting *D. melanogaster* total head lysates preparation

30-40 *D. melanogaster* adults with specific phenotype, gender, age (days after pupal eclosion) and condition were collected; after freezing in liquid nitrogen and vortexing of entire flies, separated heads were collected, counted and lysed in SDS-rich buffer (SDS 1.45%, Glycerol 20%; Tris Base 0.2 M. 2.5 µl of buffer/head) using sonication (3 pulses of 50 Watt for 5 seconds). Homogenized lysate was then centrifuged at 1000 x g for 3 seconds to separate cuticle debris from supernatant. Proteins in supernatant were collected and quantified using spectrophotometry (Implant NanoPhotometer UV/Vis). Protein content was equalized. Samples, supplied with β-mercaptoethanol 5% and bromophenol blue, were boiled at 99 °C for 5 minutes. Equal amounts of volume were resolved on SDS-PAGE. Flies of the same line were collected from different vials and the entire experiment was repeated at least 2 times.

Western Blotting and Blot quantification

Following the preparation of samples, proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and processed for Western Blotting. Primary antibodies (at concentrations mentioned above) were prepared in 3% BSA/PBS-Tween 20 0.1%, secondary antibodies at concentration 1:5000 (Invitrogen, horse peroxidase conjugated to IGG or IGM) in 5% milk/PBS-Tween 20 0.1%. For visualization membranes were incubated with Pierce ECL Western Blotting substrate (cat. No. 32106) for 2 minutes and visualized using ChemiDoc Touch Imaging System (BioRad). Blots have been quantified using Image Lab Version 5.2.1 software (BioRad).

Imaging of fluorescent eyes in *D.melanogaster* and quantification

Experimental *D. melanogaster* adults with specific phenotype, gender, age (days after pupal eclosion) and condition were kept anesthetized on a CO2 pad and their GFP fluorescent eyes were visualized using Leica MZ10 F Fluorescence stereomicroscope (GFP3 filter). 10 eyes of different flies / group were visualized and GFP fluorescence was quantified using Image J 1.48v software and expressed as corrected mean eye fluorescence (CMEF). CMEF has been calculated as: Integrated Density - (Selected Area x Mean Background Fluorescence). Statistical significance of CMEF differences analysed with 1-way ANOVA test using Graph Pad Prism Software Version 5.00.

Analysis of eye degeneration at light microscope

For each condition, at least 80 fly eyes were checked. The fraction of the eyes that showed degeneration phenotype were calculated as previously (Vos et al., 2010). For each analysed line, eyes of at least 40 flies were scored. The results were average of at least three independent experiments.

3. Results

In order to test whether and how the expression of HSPs in astrocytes might be relevant for neuroprotection in PolyQ diseases, we decided to use DNAJB6, one of the most potent human chaperones in providing protection against these diseases in different *in vitro* and *in vivo* models (Hageman et al., 2010; Kakkar et al., 2016).

In the set of experiments presented in this Chapter, we mainly aim to verify that the co-expression of the chaperone in the same neurons expressing the toxic PolyQ protein provides cell-autonomous protection in the *D. melanogaster* model of HD.

We verified whether such protective function of human DNAJB6 against PolyQ aggregation was also maintained in *D. melanogaster*. To do so, we first used the ommatidia integrity of transgenic *D. melanogaster* lines as readout for PolyQ-mediated degeneration. Hereto, the construct HttQ100-mRFP (encoding for human PolyQ-HTT exons 1-12; Weiss et al., 2012) was expressed in fly ommatidia together with human DNAJB6 (short nuclear and cytosolic isoform B; Hanai et al., 2003) and the membrane-targeted mCD8-GFP (a quantifiable fluorescent reporter of internal ommatidia integrity, established and validated in different *D.melanogaster* ommatidia-models of PolyQ diseases; Burr et al., 2014), using the *gmr* promoter driven by the Gal4-UAS expression system (Brand and Perrimon, 1993). The sole expression of HttQ100-mRFP caused a significant reduction in mCD8-GFP fluorescence in ommatidia (Fig.1A, B) and in total mCD8-GFP protein levels (Fig. 1C, D), indicating the degeneration of ommatidia. Both endpoints were alleviated by co-expression of DNAJB6 in the same cells (Fig. 1A-D), which implies a cell-autonomous protective effect of this HSP against HttQ100-mRFP toxicity. Notably, HttQ100-mRFP aggregation and mCD8-GFP levels are inversely correlated, further validating the use of this reporter, and confirming previous mechanistic findings that DNAJB6 can directly prevent the aggregation of PolyQ proteins in a cell-autonomous manner (Hageman et al., 2010; Kakkar et al., 2016). In line with the protective action against cell degeneration, DNAJB6 expression also resulted in a reduction in the amount of HttQ100-mRFP aggregates in total head lysates (Fig. 1C, D).

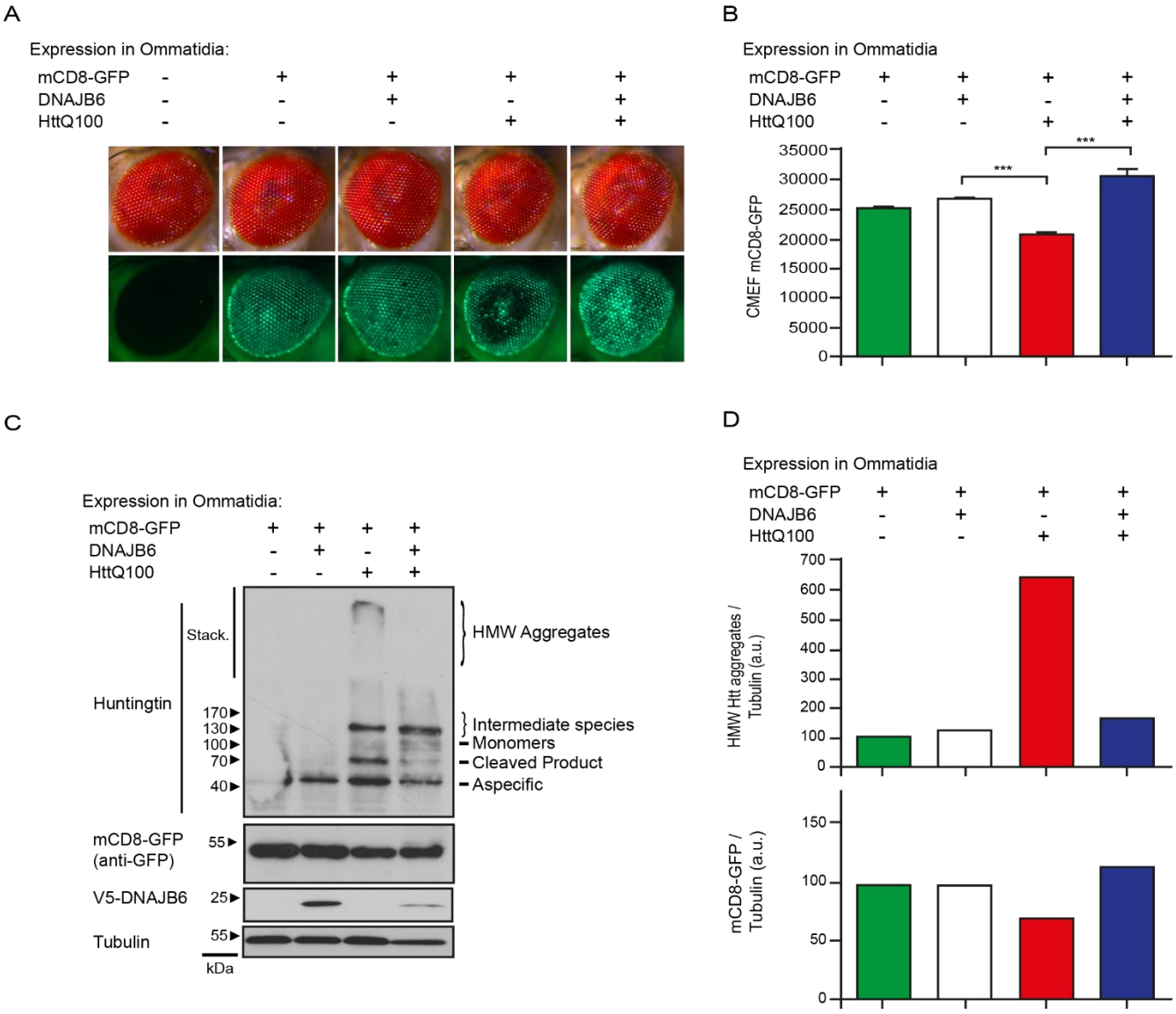

Figure 1: Protective activity of DNAJB6 against PolyQ-HTT-mediated degeneration in D. melanogaster ommatidia. A*) Representative images of eyes in 48 hours old adult male flies expressing the indicated transgenes in ommatidia. mCD8-GFP is a reporter for ommatidia integrity. Genotypes in Materials and Methods.* ***B****) Quantification of the corrected mean eye fluorescence (CMEF) for mCD8-GFP of Fig.1A. Statistical significance was analysed using 10 eyes/group with 1-way ANOVA test (SEM, ***, p<0,001).* ***C)*** *Western Blots of total head lysates of 48 hours old adult female flies expressing the indicated transgenes in ommatidia. Anti-huntingtin antibody used for detection of monomers (100 kDa), cleaved products (70 kDa), intermediate aggregated species (130-170 kDa) and HMW aggregates (stacking gel) of HttQ100-mRFP. Anti-GFP antibody used for mCD8-GFP detection. Anti-V5 antibody for (V5 tagged) DNAJB6 detection. Tubulin was used as loading control. Number of fly heads per each lysate sample and genotypes in Materials and Methods.* ***D)*** *Quantification of HMW aggregates of HttQ100-mRFP (signal in stacking gel) and mCD8-GFP of data of Fig. 1C (signal normalized on tubulin; a.u.: arbitrary units).*

Comparable data were found, in a different set of experiments, when co-expressing DNAJB6 with a truncated ataxin-3 (ATXN3) carrying an expanded PolyQ (ATXN3-Q78) (Warrick et al., 1998), responsible for spinocerebellar ataxia type 3 (OMIM:#109150) (Costa and Paulson, 2012) (Fig.2). This confirms our previous findings that the protective action of DNAJB6 is generic for the core

PolyQ-expansion (Månsson et al.,2014) and independent from regions flanking the PolyQ expansion for which it is known that they can affect the aggregation propensity of the PolyQ-containing protein (Kuiper et al., 2017). Interestingly, the neuronal expression of DNAJB6 leads to a near to complete eye protection (Fig.2), whilst expression of DNAJB6 in astrocytes of the same SCA-3 *D. melanogaster* model does not suffice to prevent depigmentation, but did attenuate progression into necrosis (Fig.3 of Chapter 5).

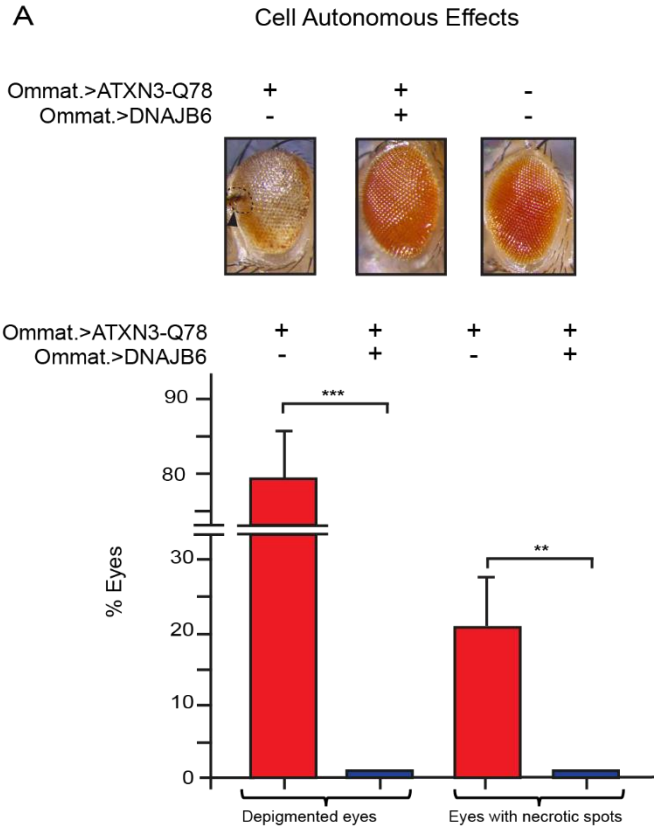

Figure 2: Cell autonomous protective activity of DNAJB6 against PolyQ-ATXN3 mediated degeneration in D. melanogaster ommatidia. A) *Cell autonomous protection by neuronal expression of DNAJB6. Top panels show representative images of eyes of 1-day old adult female flies expressing the indicated transgene. Eye degeneration is quantified as the percentage of eyes showing either depigmentation (mild degeneration) or black necrotic spots (dotted line, arrowhead) (severe degeneration). Data are compared using an unpaired t test (SD; **: P ≤ 0,01. ***: P ≤ 0,001). Genotypes in Materials and Methods.*

Next, we generated a *D. melanogaster* line expressing HttQ100-mRFP in all neurons using the validated and well-characterized pan-neuronal promoter *elav*-Gal4 (Yao et al., 1993). Western blots of total head lysates showed abundant levels of HTT monomers, cleaved products, aggregating intermediates and high molecular weight (HMW) HTT-aggregates in 5-day-old adult male (Fig. 3A) and female (Fig. 3C) flies. In 15-day-old adults, monomers and other intermediate species were remarkably decreased and HMW aggregates were still present, indicating progressive aggregation and a worsening of the HD-phenotype. Lifespan analysis showed a significant reduction in lifespan of HttQ100-mRFP expressing males (Fig. 3B), with a T50 decrease of 55% (Fig. S1A); a comparable effect has been also observed in females (Fig.3D and Fig. S1A).

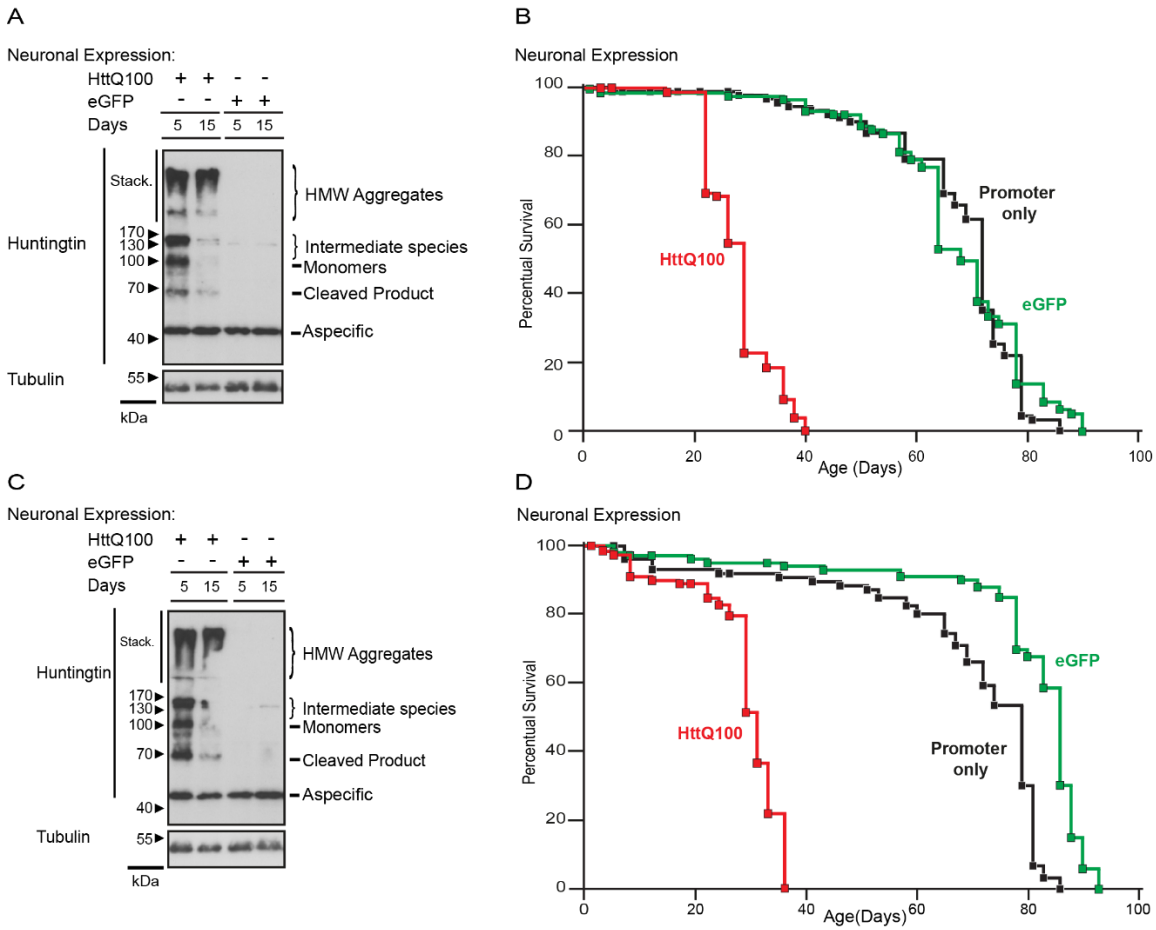

Figure 3: Pan-Neuronal expression of PolyQ-HTT causes aggregate formation and reduces lifespan in D. melanogaster. A) *Western Blots of total head lysates of 5 and 15-day-old adult male flies expressing the indicated transgenes in all neurons. Anti-huntingtin antibody for detection of monomers (100 kDa), cleaved products (70 kDa), intermediate aggregated species (130-170 kDa) and HMW aggregates (stacking gel) of HttQ100-mRFP. Tubulin was used as loading control. Number of fly heads per each lysate sample and genotypes in Materials and Methods.* ***B)*** *Lifespan of the same isogenised males flies expressing HttQ100-mRFP or control transgene (eGFP or only promoter) in all neurons. Lifespan of HttQ100-mRFP-expressing line (red curve) is significantly reduced compared to the eGFP-expressing control line (green curve). Detailed statistics, comparisons and genotypes are provided in Fig. S1A.* ***C)*** *Western Blots of total head lysates of 5 and 15-day-old adult female flies expressing the indicated transgenes in all neurons. Anti-huntingtin antibody for detection of monomers (100 kDa), cleaved products (70 kDa), intermediate aggregated species (130-170 kDa) and HMW aggregates (stacking gel) of HttQ100-mRFP. Tubulin was used as loading control. Number of fly heads per each lysate sample and genotypes in Materials and Methods.* ***D)*** *Lifespan of the same isogenised females flies expressing HttQ100-mRFP or control transgene (eGFP or only promoter) in all neurons. Lifespan of HttQ100-mRFP-expressing line (red curve) is significantly reduced compared to the eGFP-expressing control line (green curve). Detailed statistics, comparisons and genotypes are provided in Fig. S1A.*

Subsequently, we used the GAL4-UAS and LexA-LexO (Yagi et al., 2010) expression systems that can drive expression of transgenes in a completely independent and non-overlapping manner (Chapter 3). We generated lines co-expressing HttQ100-mRFP regulated by GAL4-UAS and DNAJB6 by LexA-LexO, solely in neurons (using the *elav* promoter). Neuronal expression of DNAJB6 extended the lifespan of flies co-expressing neuronal HttQ100-mRFP, increasing the T50 by 43% (Fig. 4A, Fig. S2A, S2C). The line co-expressing neuronal eGFP and HttQ100-mRFP showed a lifespan comparable to that of flies expressing neuronal HttQ100-mRFP only (compare Fig. 3B, Fig. S1A with Fig.4A, Fig.

S2A, S2C). This indicates that combined use of multiple promoters and eGFP expression was without any biological consequences, meaning that the lifespan reduction in the line co-expressing neuronal eGFP and HttQ100-mRFP of Fig.4A is primarily due to the expression of the toxic PolyQ protein. Expression of DNAJB6 in neurons of control flies did not result in lifespan modulating effects (Fig. S2B, S2C), indicating that the protection in the PolyQ model is not due to a non-specific fitness-enhancing effect of DNAJB6.

Again, in line with the previous observations (Hageman et al., 2010; Kakkar et al., 2016) and the data on *D.melanogaster* eyes-degeneration (Fig.1), we found that neuronal expression of DNAJB6 also reduced the amount of HttQ100-mRFP aggregates in total head lysates (Fig.4B, Fig. S2D). Together these data imply and confirm that DNAJB6 has a specific and cell-autonomous protective activity against HttQ100-mRFP mediated toxicity in neurons that is associated with its ability to reduce PolyQ aggregate formation (Månsson et al., 2014). Notably, brain cell degeneration due to HttQ100-mRFP toxicity might lead to reduce expression of the various transgenes during the time and disease progression, but this does not change the overall conclusion of the above described experiments.

Interestingly, we also found that neuronal expression of DNAJB6 at lower levels than those in the previous experiments in Fig.4, (using for DNAJB6, the weaker promoter *elav*-LG instead of *elav*-LHG; Yagi et al., 2010), did not lead to a cell autonomous protection in terms of lifespan and PolyQ-HTT aggregate formation in the pan-neuronal HttQ100-mRFP *D. melanogaster* model (Fig. S3A-D). These data suggest that the *in vivo* cell-autonomous protection of DNAJB6 also depends on the level of the expression of the protective chaperone, in line with previous *in vitro* findings indicating that the quantitative level of DNAJB6 is a key factor for protection and anti-aggregation activity against PolyQ proteins (Månsson et al., 2014).

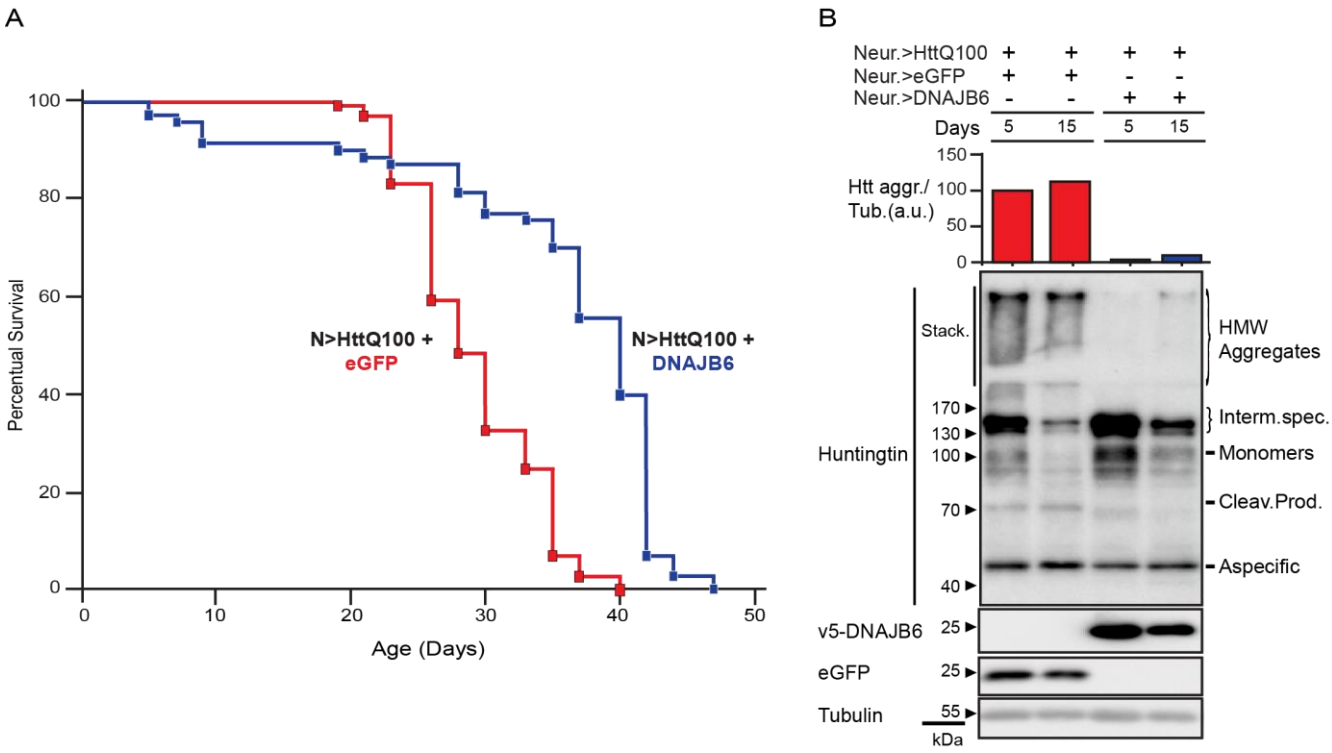

Figure 4: Effect of neuronal DNAJB6 expression on PolyQ-HTT aggregate formation and lifespan in a pan-neuronal HttQ100-mRFP D. melanogaster model. A) *Lifespan of isogenised male flies co-expressing neuronal (N>) HttQ100-mRFP and neuronal (N>) DNAJB6 or eGFP. Lifespan of DNAJB6-expressing line (blue curve) is significantly expanded compared to the control line (red curve). Additional control lines, comparisons, statistics and genotypes are provided in Fig. S2A, C.* ***B)*** *Western Blots of total head lysates of 5 and 15-day-old adult female flies (with equal transgenes expression of flies in Fig. 4A). Anti-huntingtin antibody used for detection of monomers (100 kDa), cleaved products (70 kDa), intermediate aggregated species (130-170 kDa) and HMW aggregates (stacking gel) of HttQ100-mRFP. Anti-V5 antibody for (V5 tagged) DNAJB6 detection. Anti-GFP antibody for eGFP detection. Tubulin was used as loading control. HMW aggregates of HttQ100-mRFP quantification (signal in stacking gel normalized on tubulin signal; a.u.: arbitrary units) for each line is shown. Number of fly heads per each lysate sample and genotypes in Materials and Methods. An independent repeat of the experiment is shown in Fig. S2D.*

To confirm that the expression of neuronal DNAJB6 indeed resulted in the actual and specific cell autonomous protection of neurons, we analysed the level of the neuronal marker NC82 (Wagh et al., 2006) in total fly head lysates. NC82 (Bruchpilot) is a key synaptic protein for the activity and integrity of the pre-synaptic zone in *D.melanogaster* brain (Wagh et al., 2006). NC82 is critical for a normal-evoked neurotransmitter release at the chemical synapses and, notably, the RNAi-induced-reduction of neuronal NC82 expression leads to an alteration of the normal synaptic components, to locomotor inactivity, and to unstable flight in adult *D.melanogaster* (Wagh et al., 2006). The expression level of NC82 provides therefore a direct measure of the functional fitness of the neuronal population. We found NC82 levels to be strongly decreased in 15-day-old flies solely expressing neuronal HttQ100-mRFP (Fig.S4A and S4B). This NC82-decline was alleviated in flies co-expressing neuronal DNAJB6 (cell autonomous protection, Fig. 5A, B and S4C, D), implying an overall improvement of the neuronal fitness.

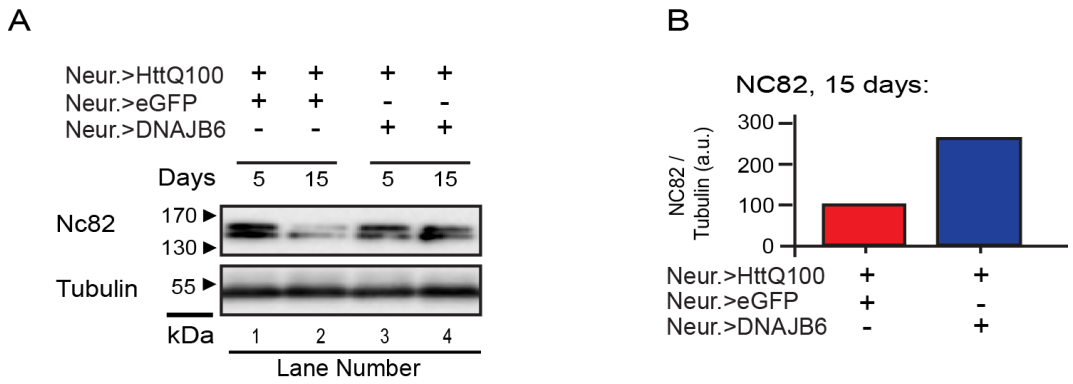

Figure 5: Effect of neuronal DNAJB6 expression in the pan-neuronal HttQ100-mRFP D. melanogaster model on overall neuronal fitness. A) *Western blots of NC82 from total head lysates of 5 and 15-day-old adult female flies co-expressing neuronal (Neur.>) HttQ100-mRFP and neuronal (Neur.>) or astrocytic (Astr.>) DNAJB6 or eGFP. Anti-NC82 antibody for NC82. Tubulin was used as loading control Number of fly heads per each lysate sample and genotypes in Materials and Methods. An independent repeat of the experiment is shown in Fig.S4C.* ***B)*** *Quantification of NC82 of data in Fig. 5A at day 15 (signal normalized on tubulin; a.u.: arbitrary units).*

4. Discussion

Here, we showed that the cell-autonomous protective function of human DNAJB6 against PolyQ aggregation are also maintained in *D. melanogaster* models of HD and SCA-3 (Chapter 3 and this Chapter).

We recapitulated and confirmed the previous findings that DNAJB6 is protective against PolyQ aggregate toxicity *in vivo*. The cell-autonomous protective effects of DNAJB6 are strongly associated with its ability to prevent the initiation of PolyQ aggregation by the core-PolyQ fragment, irrespective of regions flanking the expansion (Hageman et al., 2010; Månsson et al., 2014), for which it is known that they can affect the aggregation propensity of the PolyQ-containing protein (Kuiper et al., 2017). The suppression of PolyQ Htt aggregation via overexpression of DNAJB6 in the affected neurons of *D. melanogaster* results in a significant expansion of the lifespan and improved neuronal fitness. Noticeably, we also found that the capacity of cell-autonomous protection of DNAJB6 against PolyQ aggregation is also dependent by the expression level of the chaperone. Our and previous findings confirm that DNAJB6 is among the strongest protectors against toxicity associated with PolyQ protein aggregation. Importantly, the cell-autonomous protective capacity of DNAJB6 mainly resides in its preventive ability to suppress aggregation of PolyQ proteins and not only in alleviating the downstream consequences of aggregation (Hageman et al., 2010, Kakkar et al., 2016). All together makes DNAJB6 an interesting target against neurodegenerative protein folding diseases such as HD.

Previous findings provide insights in the mechanism of action of DNAJB6 against PolyQ aggregation that is also pertinent for the rescue that we observed in our HD and SCA3 models. Notably, DNAJB6 exerts its function by forming oligomeric complexes and by direct interaction with the PolyQ client (Kakkar et al., 2016). DNAJB6 strongly inhibits the primary nucleation step and also

perturbs the secondary nucleation in the aggregation process (Kakkar et al., 2016). Whilst the J-domain is not absolutely required, the C-terminus of the chaperone is crucial for the anti-aggregation activity (Hageman et al., 2010). Particularly, a serine/threonine (S/T)-rich region in the C-terminus of DNAJB6 is fundamental in the substrate binding (Kakkar et al., 2016, Söderberg et al., 2018). The hydroxyl groups in the side chains may reduce the primary nucleation rate of PolyQ species by competing with the hydrogen bonding necessary for formation of amyloid fibrils and beta-hairpins (Hoop et al., 2016). The J-domain of DNAJB6 allows the interaction and cooperation with HSPA/HSP70. This might link the substrate-chaperone complex to protein degradation pathways or initiate aggregate sequestration (Kumar et al., 2018).

Nonetheless, further investigations are needed to better understand the mechanisms by which DNAJB6 protects cells in a cell-autonomous manner. Insights may come by studies regarding the physiological functions of DNAJB6. As previously said, it is expressed ubiquitously and DNAJB6 orthologs are found in all metazoans analysed so far (Hageman et al., 2010). Data suggests that DNAJB6 is important for protein degradation (Izawa et al., 2000; Watson et al., 2007) and expression network analysis indicate that its expression is strongly associated with catabolic processes (Kakkar et al., 2016). DNAJB6 may therefore have a role in preventing amyloid formation due to the fragmentation of full-length proteins during such catabolic events (as it occurs in the Htt aggregation process). Consistently, DNAJB6 mutations cause a dominant heritable form of limb-girdle muscular dystrophy (Harms et al., 2012; Sarparanta et al., 2012) associated with amyloid formation in muscle biopsies (Suarez-Cedeno et al., 2014) and the downregulation of endogenous DNAJB6 enhances PolyQ aggregation in vitro (Hageman et al., 2010). Moreover, cell autonomous protection of DNAJB6 has been shown not only in PolyQ diseases (Hageman et al., 2010, Kakkar et al., 2016) but also against the toxicity of other disease-causing proteins in Alzheimer's (Månsson et al., 2014; Månsson et al., 2018) and Parkinson's (Aprile et al., 2017, Kakkar et al., 2016b). This supports the hypothesis that DNAJB6 serves as strong generic inhibitor of aggregation in cells.

Further insights into the DNAJB6 protective mechanisms will provide insights in understanding the aggregation process of these proteins and how to inhibit such process, leading to future therapeutic strategies against neurodegenerative diseases.

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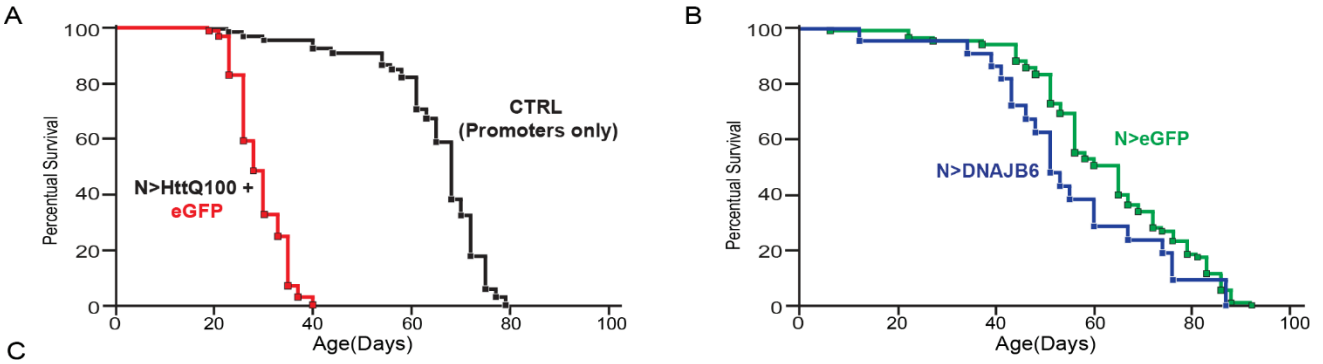
SUPPLEMENTARY DATA

A

	LINE	EXPRESSION	GENOTYPE	MEDIAN SURVIVAL	TEST 1	TEST 2
Males	Promoter only	no	*w(-); +/+; elav Gal4/+*	72	ns	ns
	eGFP	Neuronal eGFP	*w(-); UAS eGFP/+; elav Gal4/+*	71		
	HttQ100	Neuronal HttQ100-mRFP	*w(-); UAS HttQ100-mRFP/+; elav Gal4/+*	29	***, P<0,0001	***, P<0,0001
Females	Promoter only	no	*w(-); +/+; elav Gal4/+*	79	***, P<0,0001	***, P<0,0001
	eGFP	Neuronal eGFP	*w(-); UAS eGFP/+; elav Gal4/+*	86		
	HttQ100	Neuronal HttQ100-mRFP	*w(-); UAS HttQ100-mRFP/+; elav Gal4/+*	31	***, P<0,0001	***, P<0,0001

Figure S1: Statistics, comparison and genotypes of lifespan curves (male and female flies) shown Figure 3B and 3D. ***A)*** *Genotypes of lines, comparisons, and statistical analysis of lifespan curves in Fig. 3B and 3D. Statistical significance analysed using ≈100 flies/group with Log Rank Mantel-Cox test (Test 1) and Gehan-Breslow-Wilcoxon Test (Test 2).*



C

LINE	EXPRESSION	GENOTYPE	MEDIAN SURVIVAL	TEST 1	TEST 2
Ctrl (only promoters)	no	*w(-); elav LhG/+; elav Gal4/+*	68	***, P<0,0001	***, P<0,0001
N>HttQ100+eGFP	Neur.HttQ100-mRFP(Gal4-UAS)+Neur.eGFP(LhG-LexO)	*w(-); UAS HttQ100-mRFP/ elav LhG; elav Gal4/LexO eGFP*	28		
N>HttQ100+DNAJB6	Neur.HttQ100-mRFP(Gal4-UAS)+Neur.DNAJB6(LhG-LexO)	*w(-); UAS HttQ100-mRFP/ elav LhG; elav Gal4/LexO DNAJB6*	40	***, P<0,0001	***, P<0,0001
N>eGFP	Neur.eGFP (LhG-LexO)	*w(-); elav LhG/+; elav Gal4/LexO eGFP*	65	ns	*, P=0,0302
N>DNAJB6	Neur.DNAJB6 (LhG-LexO)	*w(-); elav LhG/+; elav Gal4/LexO DNAJB6*	51		

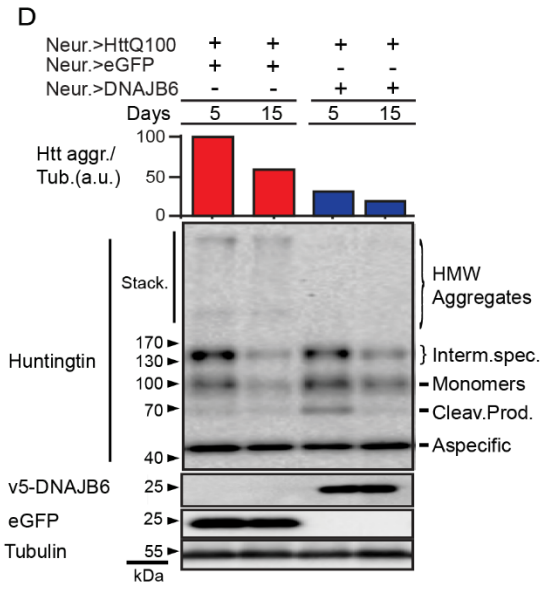
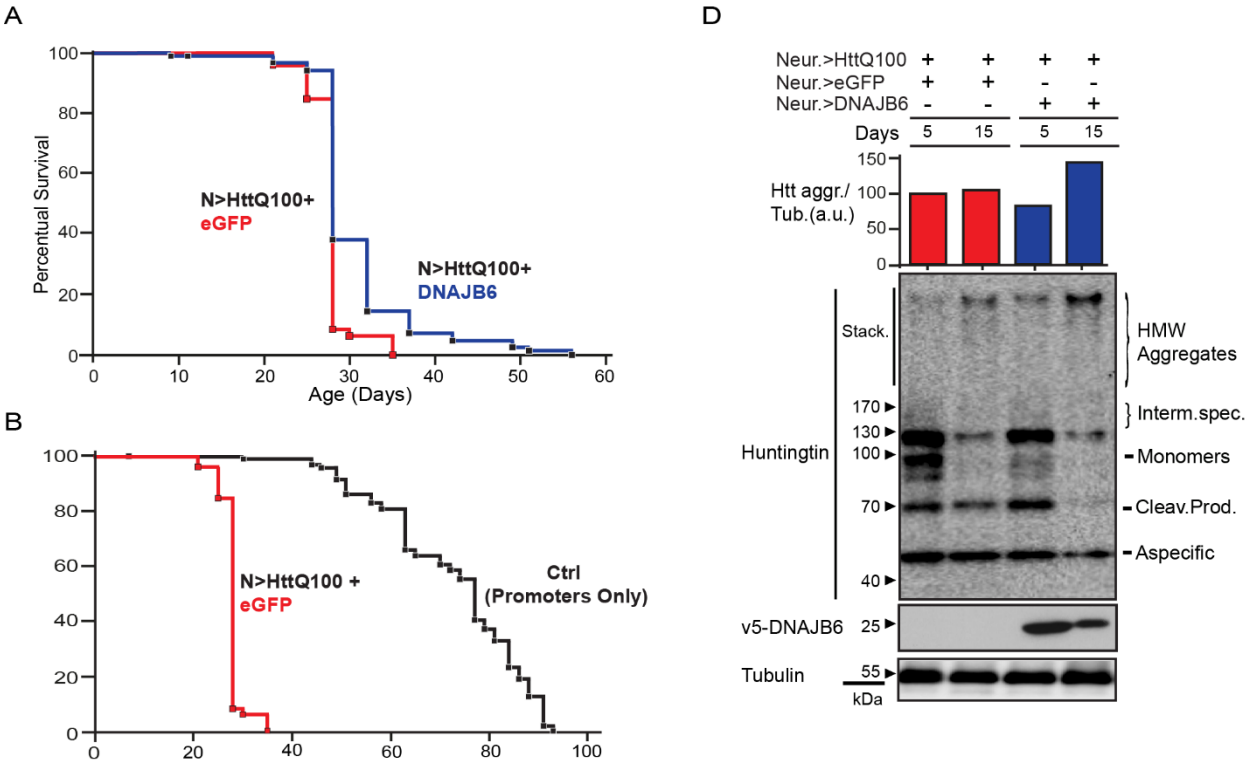


Figure S2: Lifespan analyses of control lines in Fig.4. A) *Lifespan of isogenised male flies (additional control lines of Fig. 4A) co-expressing neuronal (N>) HttQ100-mRFP and neuronal (N>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2C.* ***B)*** *Lifespan of isogenised male flies expressing neuronal (N>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2C.* ***C)*** *Genotypes of lines, comparisons, and statistical analysis of lifespan curves of Fig. 4A and S2A, S2B. Statistical significance analysed using ≈100 flies/group with Log Rank Mantel-Cox test (Test 1) and Gehan-Breslow-Wilcoxon Test (Test 2).* ***D****) Independent repeat of experiment shown in Fig. 4B.*



C

LINE	EXPRESSION	GENOTYPE	MEDIAN SURVIVAL	TEST 1	TEST 2
Ctrl (only promoters)	no	*w(-); elav LG/+; elav Gal4/+*	77		
N>HttQ100+eGFP	Neur.HttQ100-mRFP(Gal4-UAS)+Neur.eGFP(LG-LexO)	*w(-); UAS HttQ100-mRFP/ elav LG; elav Gal4/LexO eGFP*	28	***, P<0,0001	***, P<0,0001
N>HttQ100+DNAJB6	Neur.HttQ100-mRFP(Gal4-UAS)+Neur.DNAJB6(LG-LexO)	*w(-); UAS HttQ100-mRFP/ elav LG; elav Gal4/LexO DNAJB6*	28	***, P<0,0001	***, P<0,0001

Figure S3: Effect of neuronal DNAJB6 <u>moderate</u> expression on PolyQ-HTT aggregate formation and lifespan in a pan-neuronal HttQ100-mRFP D. melanogaster model. A) *Lifespan of isogenised male flies co-expressing neuronal (N>) HttQ100-mRFP and neuronal (N>) DNAJB6 or eGFP. Data shown are for moderate expression of LexO-DNAJB6/eGFP using elav-LG promoter. Genotypes: 1) HttQ100-eGFP line (red): w(-); UAS HttQ100-mRFP / elav **LG**; elav Gal4 / LexO eGFP. 2) HttQ100-DNAJB6 line (blue) w(-); UAS HttQ100-mRFP / elav **LG**; elav Gal4 / LexO DNAJB6. Additional control lines, comparisons, statistics and genotypes are provided in Fig. S3C.* ***B)*** *Lifespan of isogenised male flies (additional control lines of Fig. S3A) co-expressing neuronal (N>) HttQ100-mRFP and neuronal (N>) moderate DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S3C.* ***C)*** *Genotypes of lines, comparisons, and statistical analysis of lifespan curves of Fig. S3A-B. Statistical significance analysed using ≈100 flies/group with Log Rank Mantel-Cox test (Test 1) and Gehan-Breslow-Wilcoxon Test (Test 2).* ***D)*** *Western Blots of total head lysates of 5 and 15-day-old adult female flies (with equal transgenes expression of flies in Fig.S3A). Anti-huntingtin antibody used for detection of monomers (100 kDa), cleaved products (70 kDa), intermediate aggregated species (130-170 kDa) and HMW aggregates (stacking gel) of HttQ100-mRFP. Anti-V5 antibody for (V5 tagged) DNAJB6 detection. Anti-GFP antibody for eGFP detection. Tubulin was used as loading control. HMW aggregates of HttQ100-mRFP quantification (signal in stacking gel normalized on tubulin signal; a.u.: arbitrary units) for each line is shown.*

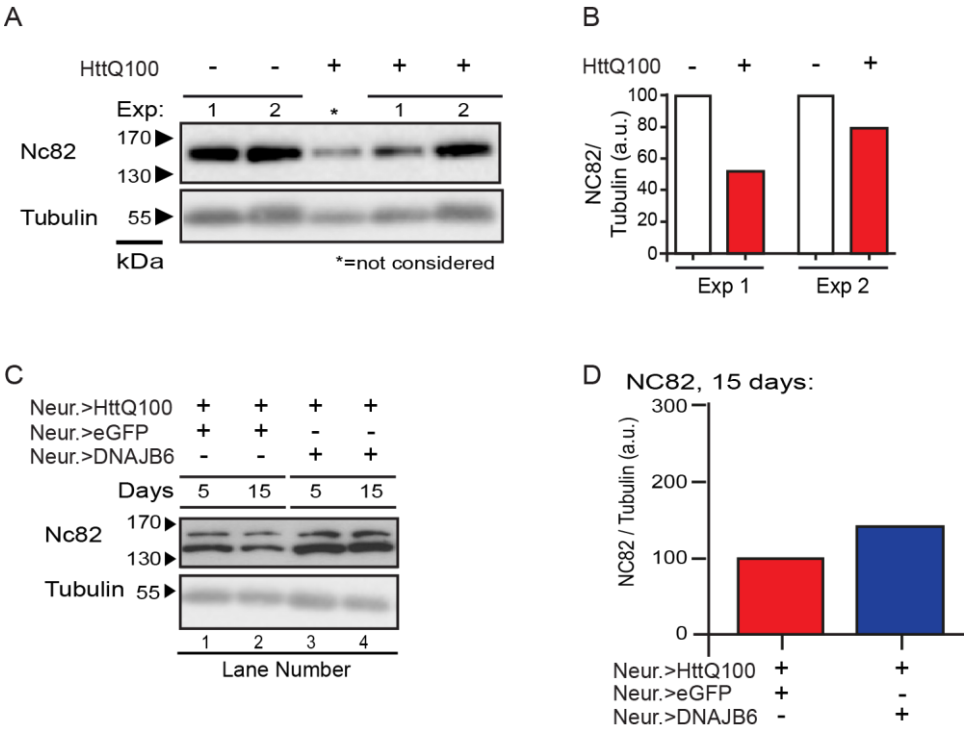


Figure S4. Supplemental experiments to Fig.5. A) *Western Blots of NC82 from total head lysates of 15-day-old adult female flies with or without neuronal HttQ100-mRFP. Anti-NC82 antibody for NC82. Tubulin was used as loading control. Number of fly heads per each lysate sample and genotypes in Materials and Methods.* ***B)*** *Quantification of NC82 for data of Fig. S4A (signal normalized on tubulin; a.u.: arbitrary units).* ***C)*** *Independent repeat of experiment shown in Fig. 5A.* ***D)*** *Quantification of NC82 for data of Fig. S4C at day 15 (signal normalized on tubulin; a.u.: arbitrary units).*

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CHAPTER 5

Astrocytic expression of the chaperone DNAJB6 results in non-cell autonomous protection in Huntington's disease

In part based on: Bason M, et al. (2019) Astrocytic expression of the chaperone DNAJB6 results in non-cell autonomous protection in Huntington's disease. Neurobiol Dis. 124:108-117.

ABSTRACT

Several neurodegenerative diseases like Huntington's, a polyglutamine (PolyQ) disease, are initiated by protein aggregation in neurons. Furthermore, these diseases are also associated with a multitude of responses in non-neuronal cells in the brain, in particular glial cells, like astrocytes. These non-neuronal responses have repeatedly been suggested to play a disease-modulating role, but how these may be exploited to delay the progression of neurodegeneration has remained unclear. Interestingly, one of the molecular changes that astrocytes undergo includes the upregulation of certain Heat Shock Proteins (HSPs) that are classically considered to maintain protein homeostasis, thus resulting in cell autonomous protection. Previously, we discovered DNAJB6, a member of the human DNAJ family, as potent cell autonomous suppressor of PolyQ aggregation and related neurodegeneration. In the Chapter 4, we confirmed that the cell autonomous protective function of human DNAJB6 against PolyQ aggregation was also maintained in *D. melanogaster*. In this Chapter, using the same cell type specific expression systems in *D. melanogaster*, we show that exclusive expression of DNAJB6 in astrocytes (that do not express PolyQ protein) can delay neurodegeneration and expands lifespan when the PolyQ protein is exclusively expressed in neurons (that do not co-express DNAJB6 themselves). This provides direct evidence for a non-cell autonomous protective role of astrocytes in PolyQ diseases.

KEYWORDS

Neurodegeneration - Polyglutamine - Aggregation - Astrocytes - Chaperones - DNAJB6- prion-like aggregate spreading

1. Introduction

Several neurodegenerative diseases (NDs), including Huntington's disease (HD), are characterized by protein aggregation in brain cells and this is thought to initiate or drive their pathology and degeneration (Kakkar et al., 2014; Kampinga and Bergink, 2016). Heat Shock Proteins (HSPs), which are fundamental regulators of the Protein Quality Control (PQC) system (Hartl et al., 2011), are considered to be protective against the aggregation of disease-related proteins and thereby capable to delay the onset of NDs (Hageman et al., 2011; Vos et al., 2010; Hageman et al., 2010; Kakkar et al., 2016a). In previous studies from our group, we identified DNAJB6, a human HSP of the DNAJ family and HSP70 co-chaperone, as a very potent and cell autonomous inhibitor of protein aggregation in different *in vitro* and *in vivo* models of Polyglutamine (PolyQ) diseases (Hageman et al., 2011; Kakkar et al., 2016b), including Huntington's disease (HD, OMIM:#143100), which is characterized by the aggregation of mutant PolyQ Huntingtin (HTT).

In the study described in the Chapter 4, we showed that human DNAJB6 provides cell autonomous protection against the toxicity of PolyQ proteins (PolyQ-HTT for HD, and PolyQ-ATXN3 for SCA3) in *D. melanogaster*. Notably, the HD model used in this study (and described in the previous Chapters) exclusively express PolyQ-HTT and DNAJB6 in the same neurons in the fly brain. In line with our previous findings, we confirmed that the DNAJB6 cell autonomous protection is associated with a reduction in the PolyQ-HTT aggregate load in fly brains.

In addition to protein aggregation, a common hallmark of nearly all aggregation-related NDs is the reactivity of astrocytes, a specific type of glial cells that normally contributes to the brain homeostasis and supports the neuronal functions (Sofroniew and Vinters, 2010). The astrocytic response during aggregation-related NDs is found in degenerating areas of the brain and is characterized by a spectrum of progressive molecular, cellular and functional changes (Sofroniew and Vinters, 2010; Ben Haim et al., 2015), which are most likely a response triggered by the neuronal damage. On one hand, the (early) astrocytic response has repeatedly been suggested to serve in a protective manner to counteract the progression of neurodegeneration. On the other hand, (chronic) astrocytic reactivity has also been suggested to be a maladaptive response that leads to disease aggravation (Sofroniew, 2009).

Interestingly, one of the molecular changes detected in astrocytes in human brains affected by neurodegeneration includes the up-regulation of certain HSPs (Durrenberger et al., 2009; Seidel et al., 2012; Wilhelmus et al., 2006; Dabir et al., 2004 and Chapter 2), including DNAJB6 (Durrenberger et al., 2009). However, the functional implications of HSPs-up-regulations for the progression of neuronal degeneration have not yet been established.

To fully explore whether and how the expression of HSPs in astrocytes contributes to neuroprotection in NDs, we generated *D.melanogaster* lines that exclusively express PolyQ-HTT in neurons whilst co-expressing human DNAJB6 in astrocytes to study non-cell autonomous effects. As explained in the previous Chapters, to generate the *D.melanogaster* lines for this study, we used a

specific combination of parental lines carrying cell-specific promoters and transgenes that allow the selective expression of PolyQ-HTT and DNAJB6 respectively in neurons and glial cells/astrocytes in the fly brain (Chapter 3). Different combination of these parental lines have been used to also generate the experimental flies used in the study presented in the previous Chapter.

Strikingly, the exclusive expression of DNAJB6 in astrocytes provides non-cell autonomous protection against progressive neuronal degeneration and prolongs organismal lifespan, although not accompanied by a reduction in the PolyQ-HTT aggregate load in the fly brains (therefore differently from what observed when the chaperone is expressed together with PolyQ-HTT in the same neurons). Rather, under these conditions, in flies that express DNAJB6 in astrocytes, a high fraction of astrocytes now contain neuronal derived PolyQ-HTT aggregates, in line with the suggestion that astrocytes might take up prion-like PolyQ-HTT aggregates species, a capacity that is enhanced by DNAJB6 expression (Brundin et al., 2010; Costanzo and Zurzolo, 2013; Ren et al., 2009; Babcock et al., 2015).

2. Materials and methods

Vectors

UAS/LexO vectors were obtained by cloning the sequences of HttQ100-mRFP (Prof. T. Littleton Group, MIT) or V5-DNAJB6 (isoform B) or eGFP (Clontech) in the multiple cloning site of pUAS *attB* or pLexO *attB* (Prof. K. Basler Group, UZH). Driver (Promoter cell-specific expression) vectors were obtained starting from the backbone of plasmids pDPP-Gal4 *attB* or pDPP-LG *attB* or pDPP-LhG *attB* (Prof. K. Basler Group, UZH). DPP promoter was substituted with the sequence of promoter *elav* (pan-neuronal, from p*Elav*-Casper vector, Prof. Liqun Luo, Stanford University), *repo* (pan-glial, from pENTRY-D-TOPO-*Repo*4.3 vector, Prof. C. Klämbt, University of Münster) or *alrm* (astrocytic, from p*Alrm*-Casper vector, Prof. M. Freeman, UMASS). All obtained vectors were sequenced. See table T1 of Chapter 3 for vectors list.

Generation of new *D. melanogaster* lines

The *D. melanogaster* lines of table T1 were obtained by injection and transformation of embryos with the above mentioned *attB* vectors, based on *attP*-site specific PhiC31 integrase system, by Best Gene Inc. injection service (https://www.thebestgene.com/HomePage.do). *D. melanogaster* lines from Bloomington Drosophila Stock Center were also used: *gmr-Gal4* (Line BDSC #1104 in Fig.3); *alrm-Gal4* (Line BDSC #67031 in Fig.3); UAS-mCD8-GFP (Line BDSC #5130); UAS-mCD8-RFP (Line BDSC #27391); UAS-ATXN3-Q78 (Line BDSC #8150 in Fig.3); *gmr-QF2* (Line BDSC #59283 in Fig.3 was a gift from C. Potter, Baltimore, MD, U.S.A.). *gmr-QF2* and QUAS-ATXN3-Q78 are based on the Q expression system in *D.melanogaster* (Potter et al., 2010). All the lines were isogenised to remove background mutations by backcrossing each of them for 6 generations with the control stock w^{1118} line. See Chapter 3 for other details.

Genotypes

- Fig. 1A and 1B*: w(-); UAS mCD8-RFP(or UAS HttQ100-mRFP) / alrm LhG; elav Gal4 / LexO eGFP.*
- Fig. 3: for non-cell autonomous rescue: *w(-),gmr-QF2; alrm-Gal4:QUAS ATXN3-Q78/ UAS DNAJB6 (or +); +/+.*
- Fig. 5A and S3A: 1) control line (red): *w(-); UAS HttQ100-mRFP / promoter(repo* or *alrm) LhG; elav Gal4 / LexO eGFP.* 2) Rescued line (blue): *w(-); UAS HttQ100-mRFP / promoter (repo* or *alrm) LhG; elav Gal4 / LexO DNAJB6.*
- Fig.7A and S4B: 1) Control (Fig. 7A, panel 1 and Fig. S4B, panels 1-3): *w(-); UAS CD8-mRFP / alrm LhG; elav Gal4 / LexO eGFP.* 2) Condition 1 (Fig. 7A, panel 2 and Fig. S4B, panels 4-6): *w(-); UAS HttQ100-mRFP / alrm LhG; elav Gal4 / LexO eGFP.* 3) Condition 2 (Fig. 7A, panel 3 and Fig. S4B, panels 7-9).
- Fig.7B and Movie M1): *w(-); UAS HttQ100-mRFP / alrm LhG; elav Gal4 / LexO DNAJB6.*
- Fig. 8A and S5A: 1) Control line (red): *w(-); UAS HttQ100-mRFP / alrm LhG; elav Gal4 / LexO eGFP.* 2) Rescued line (blue): *w(-); UAS HttQ100-mRFP / alrm LhG; elav Gal4 / LexO DNAJB6.*

Antibodies and reagents

Antibodies (dilutions are indicated in brackets for western blots (WB) and immunofluorescence (IF)) against huntingtin (Chemicon, MAB2166, WB 1:5000), eGFP (Clontech-Living Colours, cat.No.632375, WB 1:5000), α-tubulin (Sigma Aldrich, clone DM1A, WB 1:2000), V5 epitope tag in DNAJB6b (Thermo Fisher Scientific, cat. No.R960-25, WB 1:2000, IF 1:50), NC-82 (DSHB, WB 1:5000) were used. DAPI for nuclei staining (cat.No.D1306) was from Thermo Fisher Scientific. 20% SDS Solution (cat.No.1610418) was from BioRad. PBS components (NaCl cat.No.S9888, KCl cat.No.P9541, Na2HPO4 cat.No.255793, KH2PO4 cat.No.V000225), Tween-20 (cat.No.P2287), Triton X-100 (cat.No.T8787), Bovine Serum Albumin (cat.No.A2058, BSA), glycerol (cat.No.G5516), 3.7% Formaldehyde (cat.No.11-0705 SAJ), Tris base (cat.No.T1503) and β-mercaptoethanol (cat.No.M6250) were from Sigma Aldrich.

D. melanogaster stocks maintenance

All stocks and experimental flies were kept in polystyrene vials 25x95 mm filled with 8 ml/vial of solidified media (17 g/l Agar; 26 g/l Yeast; 54 g/l Sugar; 1.3 mg/l Nipagin). All experimental flies were maintained in a humidified and temperature controlled incubator at 25 °C on a 12 hours' light and 12 hours' dark cycle (Premium ICH Insect Chamber, Snijders Labs). Experimental flies, anesthetized on a CO2 pad, were selected according to their gender and phenotype by light microscope visualization.

Lifespan curves

Parental flies (5-6 females and 5-6 males) were kept in vial for 3 days and then removed. Offspring virgin flies were collected in the same 24 hours. For each analysed group, ≈100 flies of specific

gender and phenotype were collected and kept in new vials (10 flies/vial). Flies were transferred to new vials containing fresh medium every 2 days and deaths were scored daily. Statistical significance of curves differences analysed with Log rank (Mantel-Cox) test (test 1) and Gehan-Breslow-Wilcoxon test (test 2) using Graph Pad Prism Software Version 5.00. All curves comparisons were made from flies analysed in the same experiment. T50 was defined as the time point at which 50% of the initial population has died.

Western Blotting *D. melanogaster* total head lysates preparation

30-40 *D. melanogaster* adults with specific phenotype, gender, age (days after pupal eclosion) and condition were collected; after freezing in liquid nitrogen and vortexing of entire flies, separated heads were collected, counted and lysed in SDS-rich buffer (SDS 1.45%, Glycerol 20%; Tris Base 0.2 M. 2.5 µl of buffer/head) using sonication (3 pulses of 50 Watt for 5 seconds). Homogenized lysate was then centrifuged at 1000 x g for 3 seconds to separate cuticle debris from supernatant. Proteins in supernatant were collected and quantified using spectrophotometry (Implant NanoPhotometer UV/Vis). Protein content was equalized. Samples, supplied with β-mercaptoethanol 5% and bromophenol blue, were boiled at 99 °C for 5 minutes. Equal amounts of volume were resolved on SDS-PAGE. Flies of the same line were collected from different vials and the entire experiment was repeated at least 2 times.

Western Blotting and Blot quantification

Following the preparation of samples, proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane and processed for Western Blotting. Primary antibodies (at concentrations mentioned above) were prepared in 3% BSA/PBS-Tween 20 0.1%, secondary antibodies at concentration 1:5000 (Invitrogen, horse peroxidase conjugated to IGG or IGM) in 5% milk/PBS-Tween 20 0.1%. For visualization membranes were incubated with Pierce ECL Western Blotting substrate (cat. No. 32106) for 2 minutes and visualized using ChemiDoc Touch Imaging System (BioRad). Blots have been quantified using Image Lab Version 5.2.1 software (BioRad).

Analysis of eye degeneration at light microscope

For each condition, at least 80 fly eyes were checked. The fraction of the eyes that showed degeneration phenotype were calculated as previously (Vos et al., 2010). For each analysed line, eyes of at least 40 flies were scored. The results were average of at least three independent experiments.

D.melanogaster Immunofluorescence (IF), Imaging and Punctae Quantification

Brains of experimental *D. melanogaster* adults with specific phenotype, gender, age (days after pupal eclosion) and condition were dissected by light microscope visualization, in 0.1% Triton X-100 PBS 1x buffer and then fixed in 3.7% Formaldehyde, 0.1% Triton X-100 PBS 1x buffer for 20 minutes at room temperature. Staining of brains was performed by blocking not specific sites using 2% BSA,

0.1% Triton X-100 PBS 1x buffer for 30 minutes at room temperature, then incubating with primary antibody (dilution 1:50) in blocking buffer for 2 days at 4°C and finally with secondary Alexa-conjugated dyes antibody (Invitrogen, Alexa Fluor 488 goat-anti-mouse; dilution 1:500) and DAPI (final concentration 0.2 µg/µl) in blocking buffer for 2 days at 4°C, to respectively visualize primary antibodies and nuclei. Brains were mounted between glass slide and coverslip, embedded in mountant solution (CitiFluor, Agar Scientific) and visualized by confocal microscopy within 24 hours. IF images of D.melanogaster brains were captured using confocal laser scanning microscope (Leica TCS SP8). Z-stack images were obtained to check for the punctae in cells at different Z-planes. Quantification of the punctae in cells was carried out manually. Photoshop, and Image J software were used for image processing and to generate the 3D reconstruction (Movie M1). Statistical significance of values differences analysed with 1-way ANOVA test using Graph Pad Prism Software Version 5.00.

3. Results

Astrocytes are generally considered to be more resistant to PolyQ-HTT aggregation and toxicity than neurons (Jansen et al., 2014; Jansen et al., 2016) and hence PolyQ diseases are primarily thought to be due to neuronal dysfunction and death. Moreover, although chronic astrocyte reactivity is considered to be detrimental, the initial astrocytic responses have been suggested to actually counteract the progression of neurodegeneration. However, direct evidence to substantiate this hypothesis is lacking. Importantly, altered expression of several HSPs in astrocytes are amongst such molecular changes (Durrenberger et al., 2009; Seidel et al., 2012; Wilhelmus et al., 2006; Dabir et al., 2004, and Chapter 2), the relevance of which has not been demonstrated so far.

In order to test whether and how the expression of HSPs in astrocytes might be relevant for the non-cell autonomous neuroprotection in PolyQ diseases, we used D.*melanogaster* as *in vivo* model of HD, and expressed DNAJB6 in glia/astrocytes (the same short nuclear and cytosolic isoform B used in the experiments of the previous Chapter; Hanai et al., 2003) to verify its protective activity against the toxicity mediated by the expression of HttQ100-mRFP in neurons (the same human PolyQ-HTT exons 1-12 construct used in the previous Chapter; Weiss et al., 2012). To do so, we used, also for these experiments, the GAL4-UAS (Brand and Perrimon, 1993) and LexA-LexO (Yagi et al., 2010) expression systems that can drive expression of transgenes in a completely independent and non-overlapping manner.

Astrocytes have been suggested to play a key role in many aggregate-related NDs, including HD (Sofroniew and Vinters, 2010; Ben Haim et al., 2015). On the one hand, it has been suggested that the neuronal damage leads to astrocyte reactivity, which compromises their functions in nourishing and protecting neurons. On the other hand, data from rodent HD models and indirect evidence derived from human HD brains (Jansen et al., 2016) suggested that PolyQ-HTT may also be directly toxic to astrocytes, thus contributing to their chronic reactivity and to a neurodegenerative phenotype. In line with these findings and those from rodent models expressing PolyQ-HTT in astrocytes (Shin et al., 2005; Bradford et al., 2009), we observed that the selective expression of HttQ100-mRFP in the astrocytes of *D. melanogaster* (using the validated and characterized

astrocyte-specific *alrm*-Gal4 promoter; Doherty et al., 2009) reduced the lifespan of the cohort (Fig. S1A-B).

In the previous Chapter, we established that the neuronal cell-autonomous protection of human DNAJB6 is recapitulated in the *D.melanogaster* system. Here we addressed whether the up-regulation of chaperones, like DNAJB6, in astrocytes might non-cell autonomously protect neurons against PolyQ mediated degeneration. To test this hypothesis, we selectively expressed DNAJB6 in astrocytes (using the specific promoter *alrm*-LexA; Doherty et al., 2009), or in all glial cells (using the pan-glial promoter *repo*-LexA; Xiong et al., 1994), in the flies co-expressing HttQ100-mRFP in neurons (using the validated and well-characterized pan-neuronal promoter *elav*-Gal4 (Yao et al., 1993). Fig. 1 and Chapter 3).

Whereas PolyQ-HTT is ubiquitously expressed in HD human brains and rodent models of HD like R6/2 mice, in our fly model the PolyQ protein, HttQ100-mRFP, is exclusively expressed in neurons, but not in astrocytes or glial cells (Fig. 1): this enabled us to exclusively investigate whether astrocytic/glial DNAJB6 expression might exert non-cell autonomous protective effects against the toxicity mediated by the PolyQ proteins expressed in neurons. The tissue specific-expression via each of these promoters was confirmed by confocal microscopic analysis: control brains with mCD8-RFP in neurons and eGFP in astrocytes show non-overlapping staining as evidenced by diffuse mCD8-RFP fluorescence in neuronal lobes (e.g. antennal lobes, mushroom bodies), surrounded by a network of eGFP-positive astrocytes with ramified processes (Fig. 1A). Staining of brains of flies expressing neuronal HttQ100-mRFP was slightly lower. HttQ100-mRFP foci reminiscent of aggregate formation, were mainly detected in the neurons (Fig. 1B).

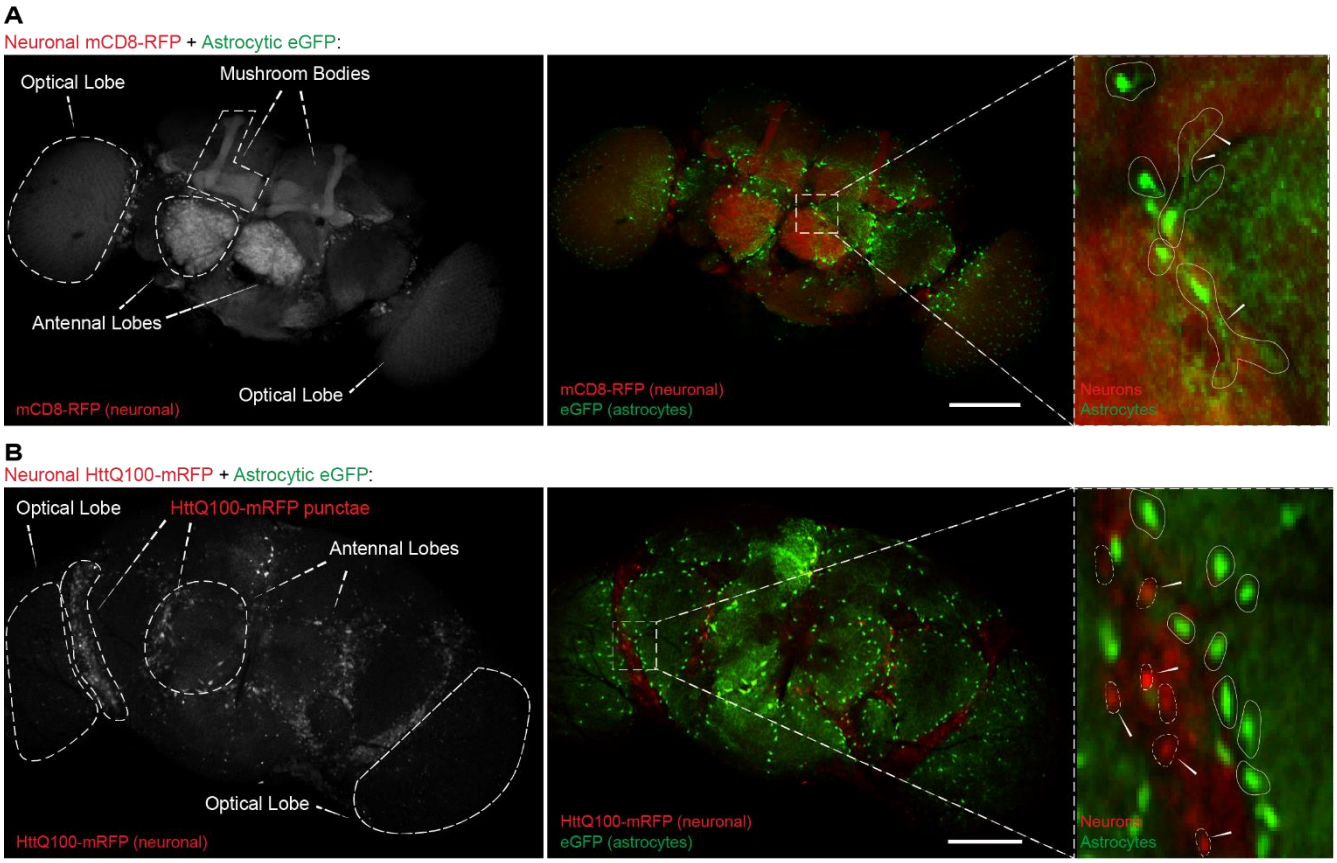

Figure 1: Co-expression of different transgenes in neurons and astrocytes of D.melanogaster brain. A,B) *Representative confocal images of brains from 15-day-old adult male flies co-expressing transgenes in different cell types using the two independent expression systems Gal4-UAS and LexA-LexO: mCD8-RFP (Fig. 1A) or HttQ100-mRFP (Fig. 1B) in all neurons and eGFP in astrocytes. Neuronal lobes and regions rich of HttQ100-mRFP punctae are shown. In the detail of the merged image of Fig. 1A, astrocytes (closed circles) with their processes (arrows) are indicated. In the detail of the merged image of Fig. 1B, neurons bodies (dashed circles) with HttQ100-mRFP punctae (arrows) and astrocytes (closed circles) are indicated. Scale bar: 100 µm. Magnification: 20x. Genotypes in Materials and Methods.*

Strikingly, the exclusive expression of DNAJB6 in all glial cells significantly expanded the lifespan of flies expressing pan-neuronal HttQ100-mRFP, increasing the T50 by 23% (Fig. 2A, Fig. S2A, E). In fact, the exclusive expression of DNAJB6 in astrocytes only resulted in a similar lifespan extension in flies expressing pan-neuronal HttQ100-mRFP (by 25%; Fig. 2B, Fig. S2B, E), indicating that the expression of DNAJB6 in this specific type of glial cells alone suffices to provide non-cell autonomous protection against PolyQ mediated toxicity. The lifespan extension in these lines is also not as pronounced as observed when DNAJB6 is expressed in neurons (in which T50 is increased by 43%; Fig. 4A, Fig. S2A, S2C of Chapter 4), suggesting that the pro-survival mechanisms mediated by DNAJB6 expression in different cell types might be mechanistically distinct and not completely equal in terms of effectiveness. Similar to what we found for exclusive DNAJB6 expression in neurons, DNAJB6 expression in all glial cells (Fig. S2C, E) or astrocytes (Fig. S2D, E) did not affect lifespan in control flies, indicating its specific effect on HttQ100-mRFP related toxicity.

We also found that both glial or astrocytic expression of DNAJB6 at lower levels than those in the previous experiments in Fig.2, (using for DNAJB6, the weaker promoters *repo*-LG or alrm-LG

respectively, instead of *repo*-LHG or alrm-LHG; Yagi et al., 2010), did not lead to a non-cell autonomous protection in terms of lifespan in the pan-neuronal HttQ100-mRFP *D. melanogaster* model (Fig. S2F, S2G and S2J for glial DNAJB6; Fig.S2H, S2I and S2J for astrocytic DNAJB6). These data suggest that, similarly to what we observe for the cell autonomous protection by neuronal DNAJB6, also the DNAJB6-mediated non-cell autonomous protection depends on the level of the expression of the protective chaperone.

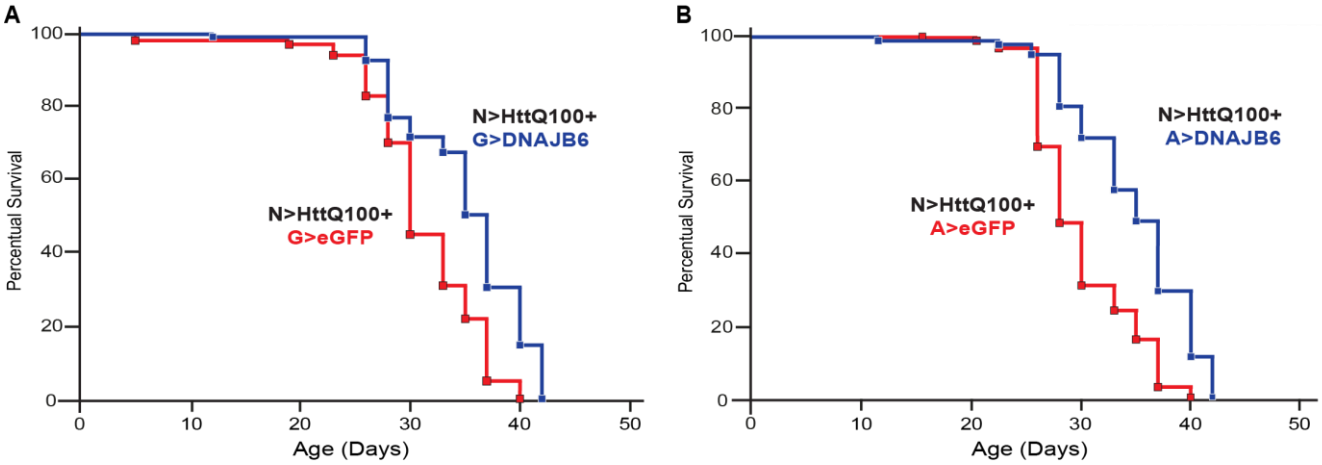

Figure 2: Effects of glial or astrocytic DNAJB6 expression on lifespan in a pan-neuronal HttQ100-mRFP D. melanogaster model. A, B) *Lifespan of isogenised male flies co-expressing neuronal (N>) HttQ100-mRFP with either glial (G>) DNAJB6 or eGFP (Fig. 2A) or astrocytic (A>) DNAJB6 or eGFP (Fig.2B). Lifespan of DNAJB6-expressing line (blue curve) is significantly expanded compared to the control line (red curve) both for Fig.2A and Fig.2B. Additional control lines, comparisons, statistics and genotypes are provided in Fig. S2A, B and E.*

In a similar approach, we also found that DNAJB6 expression in astrocytes alleviated the level of eye degeneration caused by expression of ataxin-3 (ATXN3-Q78) in fly ommatidia (Fig. 3), in a different set of experiments. This truncated ATXN3 carries an expanded PolyQ (Q78) (Warrick et al., 1998), responsible for spinocerebellar ataxia type 3 (SCA-3; OMIM:#109150) (Costa and Paulson, 2012). Interestingly, whereas the neuronal expression of DNAJB6 leads to a near to complete eye protection in the same SCA-3 *D. melanogaster* model (Fig.2, Chapter 4), the expression of DNAJB6 in astrocytes does not suffice to prevent depigmentation, but did attenuate progression into necrosis (Fig.3A).

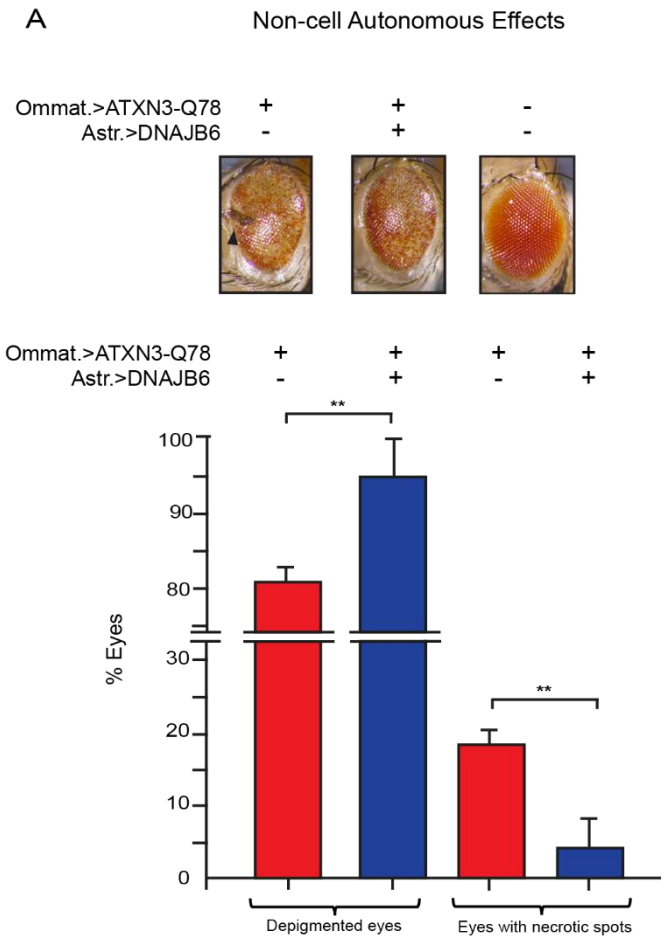

Figure 3: Non-cell autonomous protective activity of DNAJB6 against PolyQ-ATXN3 mediated degeneration in D. melanogaster ommatidia. A) *Top panels show representative images of eyes of 1-day old adult female flies expressing the indicated transgene. Eye degeneration is quantified as the percentage of eyes showing either depigmentation (mild degeneration) or black necrotic spots (dotted line, arrowhead) (severe degeneration). Data are compared using an unpaired t test (SD; **: P ≤ 0,01. ***: P ≤ 0,001). Genotypes in Materials and Methods.*

In order to investigate the mechanism underlying this non-cell autonomous protection observed in the HD model, we first considered the possibility of intercellular transmission of DNAJB6 from glial cells to neurons (Takeuchi et al., 2015) (Fig. 4).

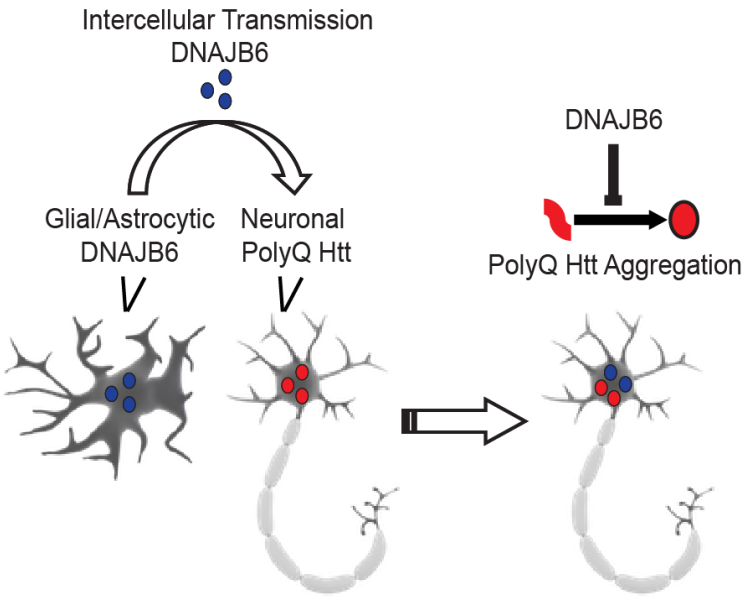

Figure 4: Schematic outline of possible intercellular transmission of DNAJB6 from glial cells/astrocytes to neurons in which the chaperone would reduce aggregation of PolyQ-HTT.

For this hypothesis to be true, such a transfer should lead to reduced aggregation of HttQ100-mRFP, similar to what we observed for the cell-autonomous protection via the expression of DNAJB6 in neurons (Fig. 4B, Fig. S2D of Chapter 4). However, DNAJB6 expression in all glial cells or astrocytes was not associated with a comparable dramatic reduction of HMW HttQ100-mRFP aggregates in total head lysates (Fig. 5A, Fig. S3A). These data confirm the specificity of the used expression systems. More importantly, these data imply that the intercellular glia-to-neurons transmission of DNAJB6 is unlikely the only or the dominant mechanism for the non-cell autonomous protective effects of DNAJB6 on the lifespan of neuronal HttQ100-mRFP flies.

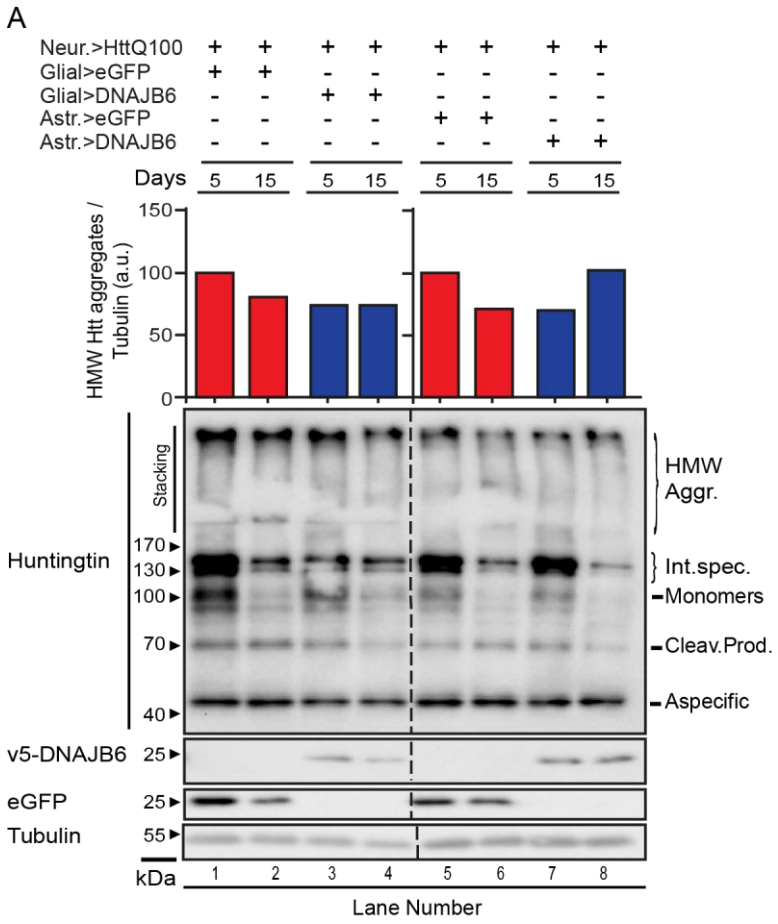

Figure 5: Effect of glial or astrocytic DNAJB6 expression in the pan-neuronal HttQ100-mRFP D. melanogaster model on aggregates formation. A) *Western Blots of total head lysates of 5 and 15-day-old adult female flies co-expressing neuronal (Neur.>) HttQ100-mRFP and glial (Glial>, lines 1-4) or astrocytic (Astr.>, lines 5-8) DNAJB6 or eGFP. Anti-huntingtin antibody used for detection of monomers (100 kDa), cleaved products (70 kDa), intermediate aggregated species (130-170 kDa) and HMW aggregates (stacking gel) of HttQ100-mRFP. Anti-V5 antibody for (V5 tagged) DNAJB6 detection. Anti-GFP antibody for eGFP detection. Tubulin was used as loading control. HMW aggregates of HttQ100-mRFP quantification (signal in stacking gel normalized on tubulin signal; a.u.: arbitrary units) for each line is shown. Number of fly heads per each lysate sample and genotypes in Materials and Methods. An independent repeat of the experiment is shown in Fig. S3A.*

In view of these results, we next investigated if other additional pro-survival and non-cell autonomous mechanisms would take place when DNAJB6 is expressed in astrocytes. It has been suggested that PolyQ-HTT aggregates may spread throughout the brain in a prion-like manner (Brundin et al., 2010; Costanzo and Zurzolo, 2013; Ren et al., 2009; Babcock et al., 2015) (Fig. 6) and that this progressive spread of disease-associated proteins contributes to the progression of the neurodegenerative process. In theory, astrocytes might restrict such spreading via actively taking up prion-like protein species, a capacity that might be limited by the progressive accumulation of toxic aggregates (Pearce et al., 2015) (Fig. 6).

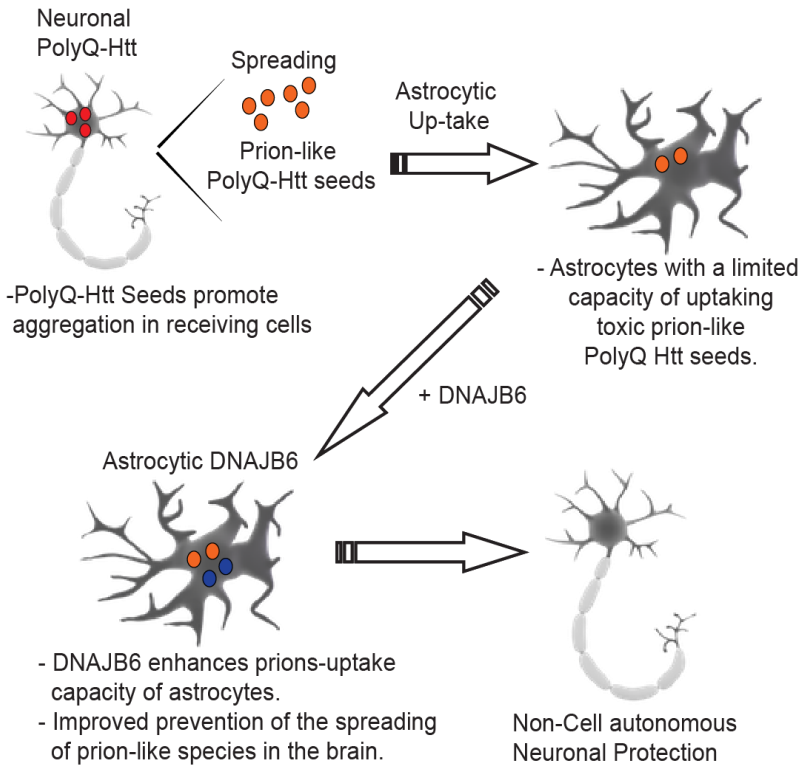

Figure 6. Schematic outline of possible spreading of PolyQ-HTT prion-like species, role of astrocytes in up-take of seeds and the non-cell autonomous protection of neurons through expression of DNAJB6 in astrocytes.

In line with this idea, confocal microscopic analysis of flies co-expressing neuronal HttQ100-mRFP and astrocytic eGFP, revealed that approximately ~10% of eGFP-expressing astrocytes, contained mRFP punctae (Fig. 7A, C; Fig. S4A, S4B). These puncta are reminiscent of protein aggregation and under all conditions are qualitatively associated with the relative amounts of insoluble proteinaceous material detected in our western analyses. This phenomenon was not due to indiscriminate protein transfer, as shown by the absence of mRFP punctae in eGFP astrocytes of the control line expressing neuronal non-aggregating membrane-bound mCD8-RFP (Fig. 7A, C; Fig. S4A, S4B). These data support the hypothesis that PolyQ HTT aggregates can indeed be transferred from neurons to astrocytes. Importantly, the combined expression of neuronal HttQ100-mRFP with astrocytic DNAJB6 resulted in a significant increase in the frequency of astrocytes with RFP punctae to ~50% (Fig. 7A-C; Fig. S4A, S4B), suggesting that DNAJB6 expression positively influenced the capacity of astrocytes of taking up such prion-like species (Fig. 6). 3-D confocal analyses confirmed that HttQ100-mRFP aggregates are indeed inside astrocytes (Fig.7B, Movie M1).

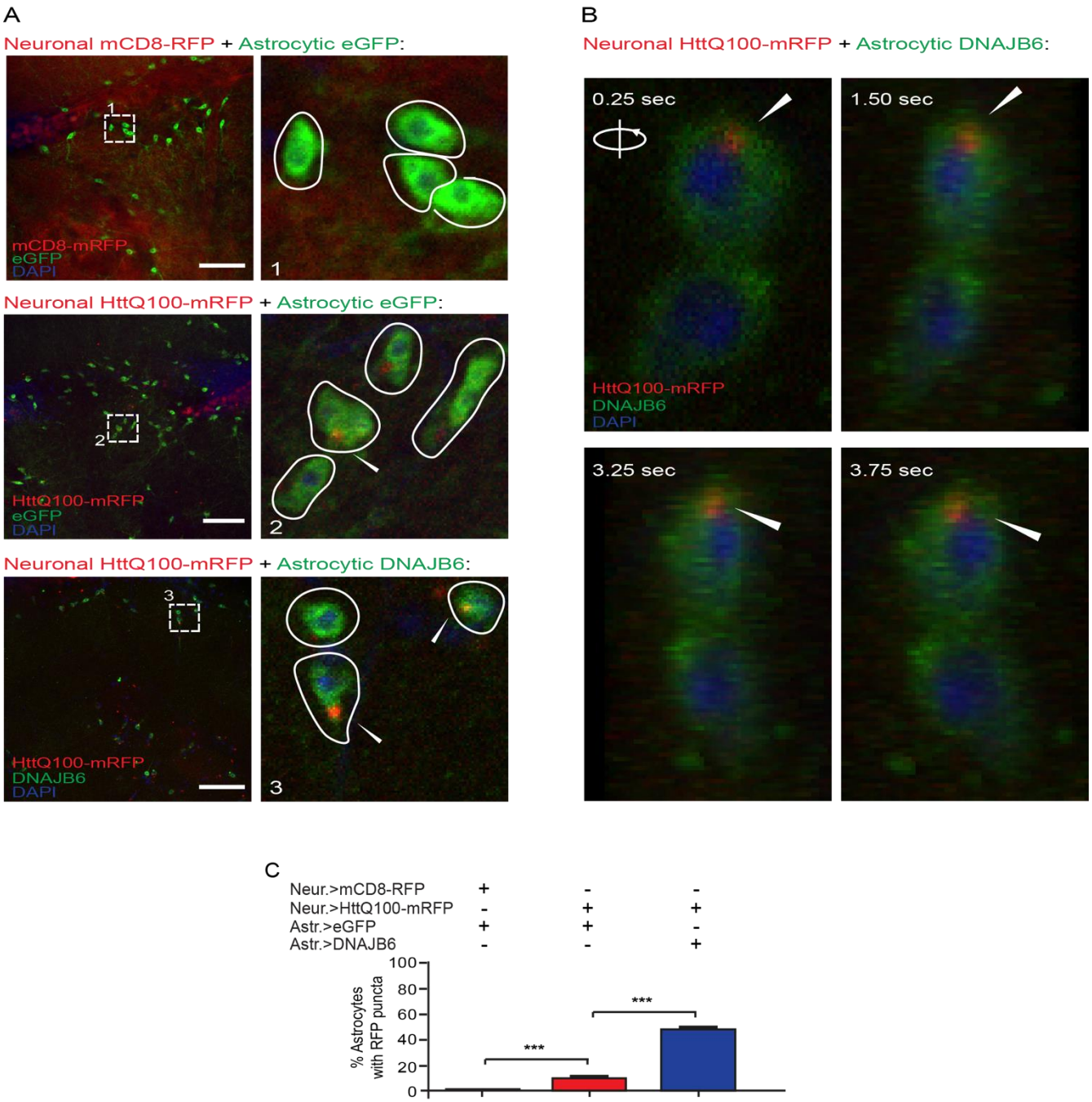

Figure 7: Effect of glial or astrocytic DNAJB6 expression in the pan-neuronal HttQ100-mRFP D. melanogaster model on aggregate distribution within the brain and overall neuronal fitness. A) *Representative confocal images of brains from 15-day-old adult male flies co-expressing neuronal mCD8-RFP or HttQ100-mRFP and astrocytic DNAJB6b or eGFP. For each indicated condition, a representative picture of the region in central brain indicated in Fig. S4A is shown. In panels 1-3, detailed images are provided showing astrocytes (closed circles) and HttQ100-mRFP punctae in astrocytes (arrows), used for the counting in Fig. 7C. Anti-V5 antibody and Alexa488 secondary antibody were used for (V5 tagged) DNAJB6 detection. Scale bar: 25 µm. Magnification: 40x. Genotypes in Materials and Methods.* ***B)*** *Frames from the representative Movie M1 (3D confocal image reconstruction) at different time points, showing astrocytes expressing DNAJB6b and containing HttQ100-mRFP puncta (arrows) from 15-day-old adult male flies expressing the indicated transgenes. Frames show the same cells from different angles. Anti-V5 antibody and Alexa488 secondary antibody were used for (V5 tagged) DNAJB6 detection. Magnification: 63x. Genotypes in Materials and Methods.* ***C)*** *Percentage of green fluorescent astrocytes containing HttQ100-mRFP puncta in a confocal*

*section of brains from flies of Fig. 7A and S4B (2 brains, 20 sections per condition). Statistical significance analysed with 1-way ANOVA test (SEM, ***, p<0,001).*

Similarly to the experiments investigating the cell autonomous protection of DNAJB6 (in the previous Chapter), to also confirm that the expression of astrocytic DNAJB6 indeed resulted in the actual and specific non-cell autonomous protection of neurons, we analysed the level of the neuronal marker NC82 (Wagh et al., 2006) in total fly head lysates. As previously explained, the expression level of NC82 provides a direct measure of the functional fitness of the neuronal population. We found NC82 levels to be strongly decreased in 15-day-old flies solely expressing neuronal HttQ100-mRFP (Fig. S4A and S4B of Chapter 4). This NC82-decline was not only alleviated in flies co-expressing neuronal DNAJB6 (cell autonomous protection, Fig. 5A, B and S4C, D of Chapter 4), but also in flies co-expressing DNAJB6 in astrocytes (non-cell autonomous protection, Fig. 8A, B and Fig. S5A, B), implying an overall improvement of overall neuronal fitness in both cases.

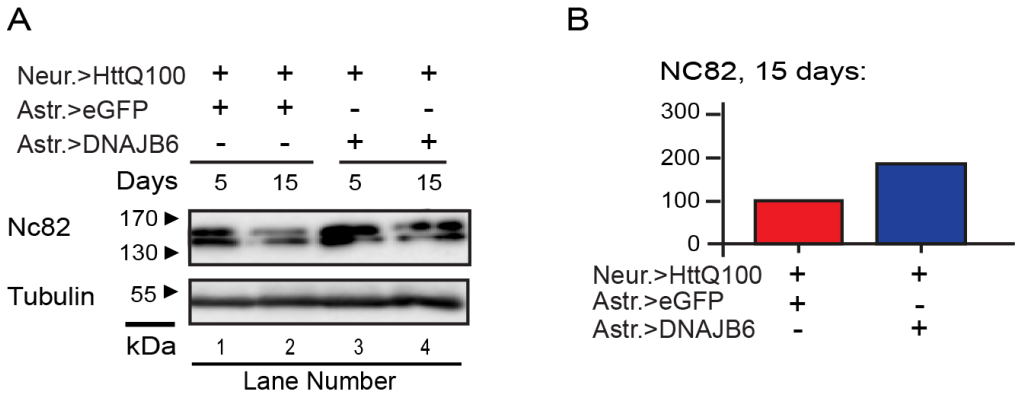

Figure 8: Effect of glial or astrocytic DNAJB6 expression in the pan-neuronal HttQ100-mRFP D. melanogaster model on overall neuronal fitness. A) *Western blots of NC82 from total head lysates of 5 and 15-day-old adult female flies co-expressing neuronal (Neur.>) HttQ100-mRFP and astrocytic (Astr.>) DNAJB6 or eGFP. Anti-NC82 antibody for NC82. Tubulin was used as loading control Number of fly heads per each lysate sample and genotypes in Materials and Methods. An independent repeat of the experiment is shown in Fig. S5A, B.* ***B)*** *Quantification of NC82 of data in Fig. 8A at day 15 (signal normalized on tubulin; a.u.: arbitrary units).*

Our experimental findings further also support the conclusion that the effects of DNAJB6 driven by *repo* or *alrm* drivers are not due to cell-autonomous effects of "leaked" neuronal expression; indeed, under the latter condition, we should have seen a reduction of HTTQ100-mRFP aggregation, which is not the case (Fig.5A, Fig. S3). Additionally, as shown in Fig. S3 of Chapter 4, we found that neuronal co-expression of HTTQ100-mRFP with DNAJB6 at lower levels compared to those in the experiment of Fig. 4 of Chapter 4 (using for DNAJB6, the weaker promoter *elav*-LG instead of *elav*-LHG respectively; Yagi et al., 2010), did not lead to cell autonomous protection. In this case, we could even detect DNAJB6 in Western blots (Fig.S3D). This makes in extremely unlikely that an eventual leakage of *repo* and *alrm* drivers, causing non-detectable expression of DNAJB6 in neurons, can be held responsible for the 24% increase in obtained life span (Fig.2A, B).

4. Discussion

Our data are a direct demonstration of a long-assumed hypothesis that glial cells, and particular astrocytes, might play a modulating role in HD, which as in many other NDs, initially and primarily affects neurons.

PolyQ-HTT is expressed in glial cells in the brain of HD patients and animal models (Jansen et al., 2016). In line with previous data from rodent models (Shin et al., 2005; Bradford et al., 2009), we confirmed that PolyQ-Htt exclusively expressed in astrocytes reduces lifespan in *D.melanogaster*. These data thus indicate that astrocytic PolyQ-HTT may contribute to HD pathology and that astrocytic damage is generally detrimental to the health of the brain, as notably observed in Alexander disease (OMIM:#203450), a genetic neurodegenerative disorder that primarily affects astrocytes by a dominant gain-of-function mutation of the *GFAP* gene (Sofroniew and Vinters, 2010; Olabarria and Goldman, 2017).

However, our data revealing neuroprotection by expression of DNAJB6 in astrocytes differ from these latter studies (Jansen et al., 2016; Shin et al., 2005; Bradford et al., 2009), as astrocytes in our *D. melanogaster* model do not express PolyQ-HTT. Still, we found that DNAJB6-expressing astrocytes provide a non-cell autonomous protective effects against degeneration of neurons expressing PolyQ-HTT. Thus, these data for the first time reveal that astrocytes empowered with sufficient chaperone activity, provided by DNAJB6, can provide protection against degeneration of neurons in HD.

DNAJB6, the chaperone used in this study, has cell-autonomous protective effects as previously shown (Kakkar et al., 2016b, Chapter 4 of this Thesis), which are strongly associated with its ability to prevent the initiation of PolyQ aggregation by the core-polyQ fragment, irrespective of regions flanking the expansion (Hageman et al., 2010; Månsson et al., 2014), for which it is known that they can affect the aggregation propensity of the PolyQ-containing protein (Kuiper et al., 2017). Our findings for both in vivo PolyQ HTT and PolyQ ATXN3 fly models show that also not only the cell autonomous, but also the non-cell autonomous effects of DNAJB6 are generic for PolyQ proteins.

Interestingly, the non-cell autonomous protection evoked by DNAJB6 expression in astrocytes is not associated with a reduction in PolyQ-HTT aggregation. Although it has been suggested that DNAJs and other HSPs can be transmitted between cells via exosomes (Takeuchi et al., 2015), the absence of an effect on total aggregate formation in our study implies that additional mechanisms may underlie the DNAJB6-mediated non-cell autonomous protection.

Despite the use of well-established promoters and two independent expression systems, it could still be argued that low levels of neuronal DNAJB6, due to an eventual leakage of the *repo*-LexA and *alrm*-LexA promoters and beyond detection limit of our microscopic analyses, could have been responsible for the observed protection evoked by DNAJB6 expression in astrocytes. However, using a weaker *elav* promoter to drive neuronal DNAJB6 expression than those used in the presented data (i.e. using the *elav*-LG instead of the *elav*-LhG promoter (Chapter 3 of this Thesis and Yagi et al., 2010)) we found no significant cell autonomous protection, whereas we could still detect neuronal

DNAJB6 expression (Fig. S3 of Chapter 4). This implies that is it highly unlikely that the observed protection by astrocytic DNAJB6 expression is due to leakage of the *repo*-LexA and *alrm*-LexA promoters.

This implies that the observed non-cell autonomous protection via astrocytic DNAJB6 expression is directly due to an activity of the chaperone in the astrocytes enhancing its fitness and function. We hypothesize such may delay the trans-cellular spreading of PolyQ HTT aggregates in the *D.melanogaster* brain. Cellular experiments have indeed demonstrated that PolyQ-HTT aggregates can enter cells where they can initiate intracellular seeding (Kakkar et al., 2016b; Ren et al., 2009) and can spread between neurons in the *D.melanogaster* brain (Babcock et al., 2015). Our data show that, in the same organismal system, these neuronal PolyQ-HTT aggregates can end up in astrocytes in agreement with observations by Pearce and colleagues (Pearce et al., 2015), using axotomised neurons. The uptake of neuron-derived aggregates could imply that astrocytes act as a "reservoir" for these toxic prion-like species, thereby preventing their neuron-to-neuron spreading in the brain and hence delaying the progression of neurodegeneration. The prion-reservoir capacity of astrocytes is likely limited by the toxicity of captured aggregates. However, the upregulation of chaperones like DNAJB6 may enhance this capacity, which is supported by our data showing that the frequency of astrocytes with inclusions is increased in case of DNAJB6 overexpression. This provokes the speculation that DNAJB6 can positively influence the prion-reservoir capacity of astrocytes and their ability to prevent the spreading of prion-like species in the brain, via its protective functions against PolyQ-HTT toxicity (Hageman et al., 2010). The increased prion-reservoir capacity of astrocytes ultimately results in an improvement of overall neuronal fitness and in an increased lifespan of PolyQ-HTT *D.melanogaster* model (Fig.6).

An alteration in neuroinflammation could be an additional contributor to the enhanced neuroprotection due to the DNAJB6 expression in astrocytes, which hereby might promote a more neuroprotective or less neurotoxic pathway. Indeed, astrocytic activation has been claimed to have both positive and negative effects on the progression of PolyQ diseases (Sofroniew and Vinters, 2010).

In conclusion, our findings support the hypothesis that increasing astrocyte fitness and functions by chaperones like DNAJB6 (tentatively by increasing their prion-reservoir capacity) has protective significance in HD and possibly other protein aggregation-related NDs which show a pathology associated with these prion-like species such as Alzheimer's (OMIM:#104300) and Parkinson's (OMIM:#168601) (Brundin et al.,2010; Costanzo and Zurzolo et al., 2013).

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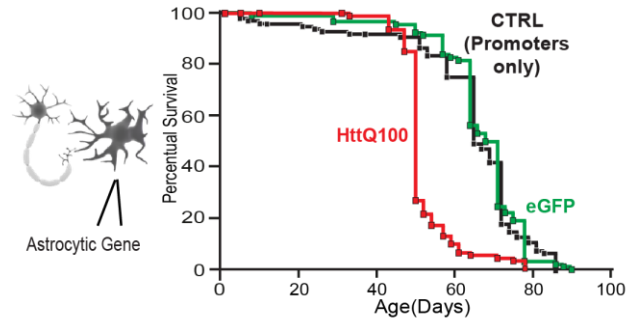
SUPPLEMENTARY DATA

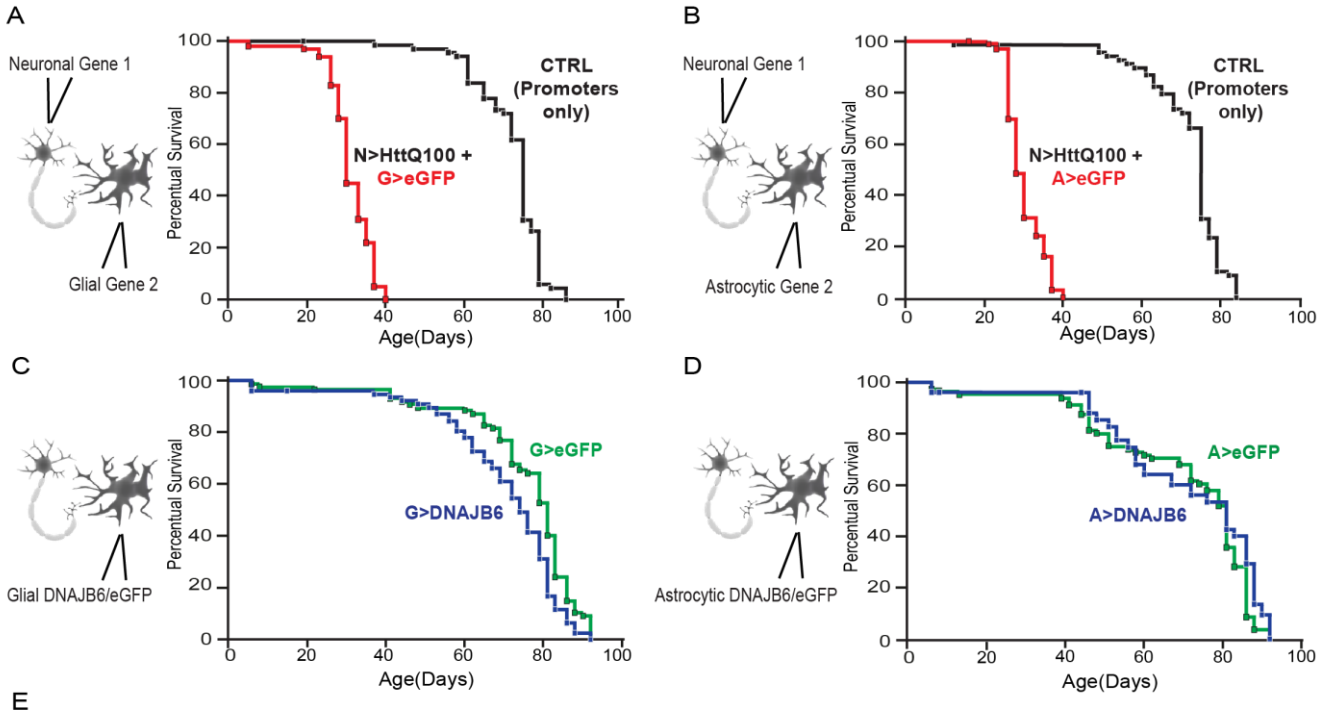
A



B

LINE	EXPRESSION	GENOTYPE	MEDIAN SURVIVAL	TEST 1	TEST 2
Ctrl (only promoters)	no	*w(-); +/+; alrm Gal4/+*	65	ns	ns
eGFP	Astrocytic eGFP (Gal4-UAS)	*w(-); UAS eGFP/+; alrm Gal4/+*	69,5		
HttQ100	Astrocytic HttQ100-mRFP (Gal4-UAS)	*w(-); UAS HttQ100-mRFP/+; alrm Gal4/+*	50	***, P<0,0001	***, P<0,0001

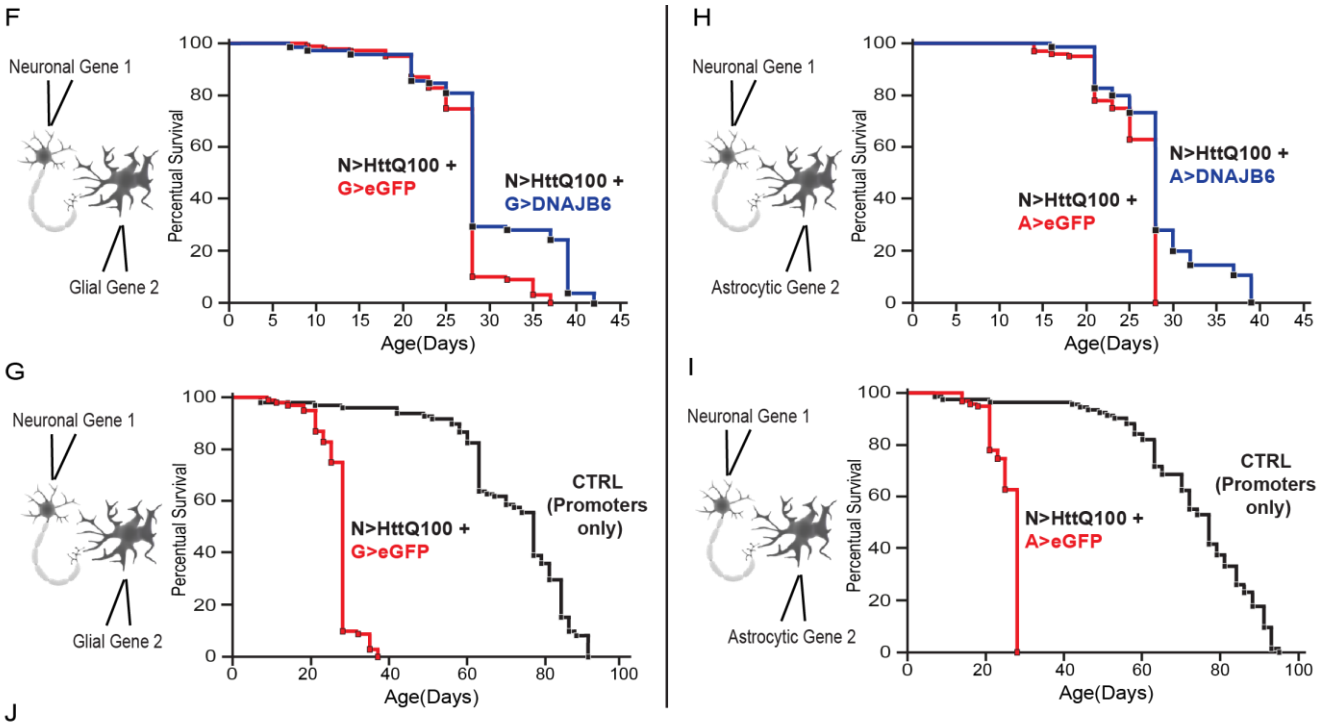
Figure S1. Lifespan analyses of D.melanogaster line expressing PolyQ HTT in astrocytes. A) *Lifespan of isogenised male expressing HttQ100-mRFP or control transgene (eGFP or only promoter) in all astrocytes. Detailed statistics, comparisons and genotypes are shown in Fig.S1B.* ***B)*** *Genotypes of lines, comparisons, and statistical analysis of lifespan curves of Fig.S1A. Statistical significance analysed using ≈100 flies/group with Log Rank Mantel-Cox test (Test 1) and Gehan-Breslow-Wilcoxon Test (Test 2).*



E

LINE	EXPRESSION	GENOTYPE	MEDIAN SURVIVAL	TEST 1	TEST 2
Ctrl (only promoters)	no	*w(-); repo LhG/+; elav Gal4/+*	75		
N>HttQ100+G>eGFP	Neur.HttQ100-mRFP(Gal4-UAS)+Glial eGFP(LhG-LexO)	*w(-); UAS HttQ100-mRFP/ repo LhG; elav Gal4/LexO eGFP*	30	***, P<0,0001	***, P<0,0001
N>HttQ100+G>DNAJB6	Neur.HttQ100-mRFP(Gal4-UAS)+Glial DNAJB6(LhG-LexO)	*w(-); UAS HttQ100-mRFP/ repo LhG; elav Gal4/LexO DNAJB6*	37	***, P<0,0001	***, P<0,0001
Ctrl (only promoters)	no	*w(-); alrm LhG/+; elav Gal4/+*	75		
N>HttQ100+A>eGFP	Neur.HttQ100-mRFP(Gal4-UAS)+Astr. eGFP(LhG-LexO)	*w(-); UAS HttQ100-mRFP/ alrm LhG; elav Gal4/LexO eGFP*	28	***, P<0,0001	***, P<0,0001
N>HttQ100+A>DNAJB6	Neur.HttQ100-mRFP(Gal4-UAS)+Astr. DNAJB6(LhG-LexO)	*w(-); UAS HttQ100-mRFP/ alrm LhG; elav Gal4/LexO DNAJB6*	35	***, P<0,0001	***, P<0,0001
G>eGFP	Glial eGFP (LhG-LexO)	*w(-); repo LhG/+; elav Gal4/LexO eGFP*	81		
G>DNAJB6	Glial DNAJB6 (LhG-LexO)	*w(-); repo LhG/+; elav Gal4/LexO DNAJB6*	74	**, P=0,0014	**, P=0,0021
A>eGFP	Astrocytic eGFP (LhG-LexO)	*w(-); alrm LhG/+; elav Gal4/LexO eGFP*	81		
A>DNAJB6	Astrocytic DNAJB6 (LhG-LexO)	*w(-); alrm LhG/+; elav Gal4/LexO DNAJB6*	81	ns	ns

Figure S2 (A-E). Lifespan analyses of control lines depicted in Fig. 2. A) *Lifespan of isogenised male flies (additional control lines of Fig. 2A) co-expressing neuronal (N>) HttQ100-mRFP and glial (G>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2E.* ***B)*** *Lifespan of isogenised male flies (additional control lines of Fig. 2B) co-expressing neuronal (N>) HttQ100-mRFP and astrocytic (A>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2E.* ***C)*** *Lifespan of isogenised male flies expressing glial (G>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2E.* ***D)*** *Lifespan of isogenised male flies expressing astrocytic (A>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2E.* ***E)*** *Genotypes of lines, comparisons, and statistical analysis of lifespan curves of: Fig. 2A and S2A; Fig. 2B and S2B; Fig. S3C; Fig. S3D. Statistical significance analysed using ≈100 flies/group with Log Rank Mantel-Cox test (Test 1) and Gehan-Breslow-Wilcoxon Test (Test 2).*



J

LINE	EXPRESSION	GENOTYPE	MEDIAN SURVIVAL	TEST 1	TEST 2
Ctrl (only promoters)	no	*w(-); repo LG/+; elav Gal4/+*	77		
N>HttQ100+G>eGFP	Neur.HttQ100-mRFP(Gal4-UAS)+Glial eGFP(LG-LexO)	*w(-); UAS HttQ100-mRFP/ repo LG; elav Gal4/LexO eGFP*	28	***, P<0,0001	***, P<0,0001
N>HttQ100+G>DNAJB6	Neur.HttQ100-mRFP(Gal4-UAS)+Glial DNAJB6(LG-LexO)	*w(-); UAS HttQ100-mRFP/ repo LG; elav Gal4/LexO DNAJB6*	28	***, P<0,0001	**
Ctrl (only promoters)	no	*w(-); alrm LG/+; elav Gal4/+*	77		
N>HttQ100+A>eGFP	Neur.HttQ100-mRFP(Gal4-UAS)+Astr. eGFP(LG-LexO)	*w(-); UAS HttQ100-mRFP/ alrm LG; elav Gal4/LexO eGFP*	28	***, P<0,0001	***, P<0,0001
N>HttQ100+A>DNAJB6	Neur.HttQ100-mRFP(Gal4-UAS)+Astr. DNAJB6(LG-LexO)	*w(-); UAS HttQ100-mRFP/ alrm LG; elav Gal4/LexO DNAJB6*	28	***, P<0,0001	***, P=0.0005

Figure S2 (F-J). Lifespan analyses of lines with <u>moderate</u> expression of glial or astrocytic DNAJB6 in the pan-neuronal HttQ100-mRFP D. melanogaster model. F) *Lifespan of isogenized male flies co-expressing neuronal (N>) HttQ100-mRFP with glial (G>) DNAJB6 or eGFP. Data shown are for moderate expression of LexO-DNAJB6/eGFP using repo-LG promoter. Additional control lines, comparisons, statistics and genotypes are provided in Fig.S2G and J.* ***G)*** *Lifespan of isogenized male flies (additional control lines of Fig. S2F) co-expressing neuronal (N>) HttQ100-mRFP and glial (G>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig.S2J.* ***H)*** *Lifespan of isogenized male flies co-expressing neuronal (N>) HttQ100-mRFP with astrocytic (A>) DNAJB6 or eGFP. Data shown are for moderate expression of LexO-DNAJB6/eGFP using alrm-LG promoter. Additional control lines, comparisons, statistics and genotypes are provided in Fig.S2I and J.* ***I)*** *Lifespan of isogenized male flies (additional control lines of Fig. S2H) co-expressing neuronal (N>) HttQ100-mRFP and astrocytic (A>) DNAJB6 or eGFP. Detailed statistics, comparisons and genotypes are shown in Fig. S2J.* ***J)*** *Genotypes of lines, comparisons, and statistical analysis of lifespan curves of Fig. S2F-G and S2H-I. Statistical significance analyzed using ≈100 flies/group with Log rank Mantel-Cox test (Test 1) and Gehan-Breslow-Wilcoxon Test (Test 2).*

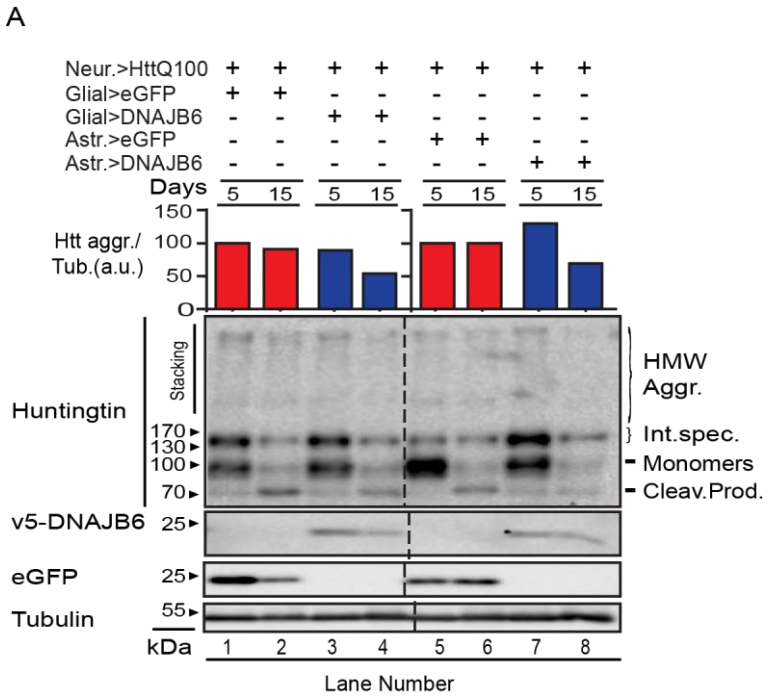


Figure S3. Effect of glial or astrocytic DNAJB6 expression in the pan-neuronal HttQ100-mRFP D. melanogaster model on aggregates formation. A) *Independent repeat of experiment shown in Fig. 5A.*

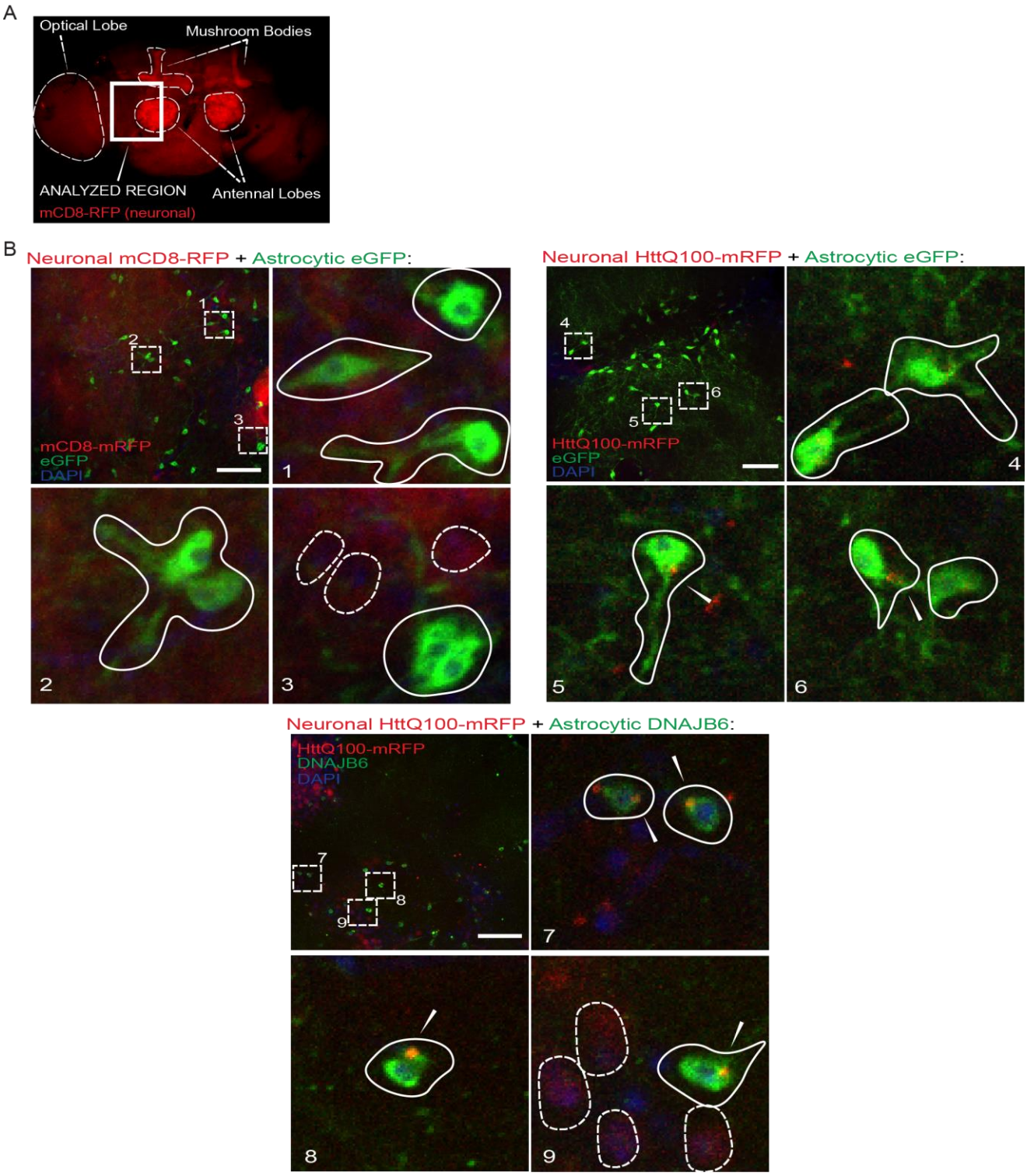


Figure S4. Supplemental experiments to Fig.7. A) *Representative confocal image of D. melanogaster brain (expressing pan-neuronal mCD8-RFP, via elav), indicating the different neuronal lobes and highlighting the central brain region analysed for data of Fig. 7A, S4B, 7B and 7C.* ***B)*** *Representative confocal images of a repeat of the experiment shown in Fig.7A. In panels 1-9, detailed images are provided showing neurons (dashed circles), astrocytes (closed circles) and HttQ100-mRFP punctae in astrocytes (arrows).*

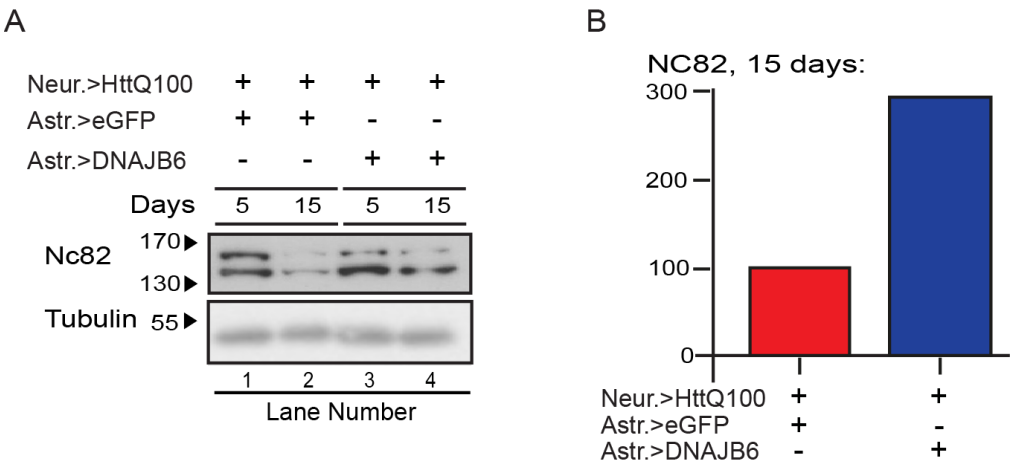


Figure S5. Supplemental experiments to Fig.8. A) Independent repeat of experiment shown in Fig. 8A, B. **I)** Quantification of NC82 for data of Fig. S5A at day 15 (signal normalized on tubulin; a.u.: arbitrary units).

MOVIE M1

M1) *Representative 3D reconstruction from confocal microscopic imaging showing astrocytes expressing DNAJB6b (green fluorescence) with HttQ100-mRFP puncta. Sample from 15-day-old adult male flies expressing neuronal HttQ100-mRFP and astrocytic DNAJB6b. Anti-V5 antibody and Alexa488 secondary antibody were used for (V5 tagged) DNAJB6 detection. DAPI was used for nuclei detection. Magnification: 63x. Genotypes in Materials and Methods. Available here:*
https://www.sciencedirect.com/science/article/pii/S0969996118304327?via%3Dihub#ec0005

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CHAPTER 6
DISCUSSION

1. Our findings and open questions

In this research, we generated and validated a *D.melanogaster* model that allows the independent and non-overlapping expression of two different transgenes of interest in neurons and astrocytes (or all glial cells) of the fly brain (Chapter 3). This model was developed for the *in vivo* investigation of the mutual interactions between neurons and astrocytes in the brain. In particular, we used this model to investigate the protective role of chaperones, and in particular both cell-autonomous and non-cell autonomous protective functions of the human chaperone DNAJB6 against Huntington's disease (HD) (Chapters 4 and 5).

First, we showed that the previously observed cell-autonomous protective function of human DNAJB6 against PolyQ aggregation and toxicity (Hageman et al., 2010; Månsson et al., 2014, Kakkar et al., 2016) can be recapitulated in *D. melanogaster* models of HD (Chapter 4). We confirmed that DNAJB6 reduces PolyQ Huntingtin (Htt) aggregate formation and toxicity and improves neuronal fitness *in vivo,* consequently leading to a significant expansion of the lifespan of the flies expressing Htt in neurons. Most strikingly, we found that also the expression of DNAJB6 only in astrocytes leads to an expansion of the lifespan of flies expressing Htt in neurons, suggesting a non-cell autonomous protective activity of the chaperone in our *Drosophila* HD model (Chapter 5).

These main findings raises a number of new questions and perspectives, as described below:

1. Our data show that the non-cell autonomous protection of astrocytic DNAJB6 does not result in the same magnitude of lifespan extension as when DNAJB6 is directly co-expressed with PolyQ Htt in neurons, implying that direct cell-autonomous neuronal protection has the strongest effects. The relevance of these findings will be discussed in section 1.1 of this Chapter.

2. The neuronal PolyQ-HTT aggregates can trans-cellularly spread in the *D.melanogaster* brain in a prion-like manner and can end up in astrocytes. We hypothesised that the uptake of neuron-derived aggregates could imply that astrocytes act as a "reservoir" for these toxic prion-like species, thereby preventing their neuron-to-neuron spreading in the brain and hence delaying the progression of neurodegeneration. However, this prion-reservoir capacity is likely limited by the toxicity of captured aggregates. Possible mechanisms for PolyQ HTT prionoids entry in astrocytes will be discussed in section 1.2 of this Chapter.

3. Unlike for cell autonomous effects, the non-cell autonomous protection evoked by DNAJB6 expression in astrocytes is not associated with a reduction in the load of total PolyQ-HTT aggregation in the brain of our HD *Drosophila* model , implying that the DNAJB6 transmission from astrocytes to neurons (such as via exosomes) is unlikely. Although not supported by our data, the chaperone transmission from astrocytes to neurons still remains an intriguing mechanisms for non-cell autonomous protection in HD that might provide possible therapeutical options (section 1.3 of this Chapter).

4. In our HD *Drosophila* model, the frequency of astrocytes with inclusions is increased in case of DNAJB6 overexpression, which suggests that DNAJB6 may have enhanced the "reservoir"

capacity of astrocytes and their ability to prevent the spreading of prion-like species in the brain. How can DNAJB6 protect astrocytes from the toxicity mediated by PolyQ Htt prionoids (section 1.4 of this Chapter)?

5. Expression of DNAJB6 in all glial cells did not provide a longer extension of the lifespan of HD flies than DNAJB6 expression only in astrocytes, suggesting that astrocytes are the key players in the non-cell autonomous protection mediated by DNAJB6. Nonetheless our data does not exclude that other glial cells, such as microglia, might have an important role, together with astrocytes, in the non-cell autonomous protection against PolyQ Htt prionoids (section 1.5 of this Chapter).

1.1 - Cell-autonomous protection of DNAJB6 is more effective

Our data reveal that the cell-autonomous protection of neuronal DNAJB6 is more effective compared to the non-cell autonomous protection of astrocytic DNAJB6 in the HD *Drosophila* model expressing PolyQ Htt in neurons. This finding strongly supports the idea that PolyQ diseases are primarily neuronal diseases. Nonetheless, our data open to the possibility that the modulation of the chaperonome in astrocytes might be an important rearguard in providing protection. This is idea is also substantiated by the crucial role of astrocytes in protecting neurons (Section 3.1 and 3.2 of Chapter 2), and by their different capacity to handle toxic PolyQ aggregates (Sections 3.3 and 3.4 of Chapter 2).

1.2 - PolyQ Htt prionoids spreading and the role of astrocytes

Our data substantiate the interesting previous evidences showing that mutant PolyQ Htt aggregates might behave like prionoids (Scheckel et al., 2018) in line with several *in vitro* and *in vivo* studies and findings in patients based on the progression patterns of the polyQ diseases within the brains of poly Q patients (see Figure 6 of Chapter 2 and references in table 1 of Chapter 2, section 2.6). Being prionoids, the PolyQ Htt aggregates are capable of seeding, therefore of elongating by the recruitment of soluble polypeptide chains and of fragmenting, to generate additional elongation sites and amplify aggregation. Importantly, these aggregate species can recruit the normal Htt protein during the seeding process (Kazantsev et al. 1999, Busch et al. 2003; Ren et al. 2009; Trevino et al. 2012; Holmes et al. 2013; Tan et al. 2015; Ruiz-Alrandis et al. 2016).

If the acceptor cells have a higher capability to cope against the PolyQ HTT toxicity, aggregation and seeding capacities, they might be capable to interfere with the prion-like spreading of toxic species and slow down the pathological processes.

In our model, astrocytes serve as acceptor cells: boosting their resistance against the up taken PolyQ Htt aggregates, through the overexpression of the protective chaperone DNAJB6, might slow down the pathological processes in HD.

An important open question is how PolyQ Htt prionoids can enter in astrocytes. Several mechanisms have been described for the spreading of PolyQ Htt prionoids in the brain:

1. Tunnelling nanotubes (TNTs)
2. Endocytosis and phagocytosis

3. Exosomes and exophers
4. Direct penetration of the plasma membrane
5. Transynaptic propagation

Altough further investigations are needed, all these mechanisms might be involved in the PolyQ Htt spreading between cells that we also observe in our HD model. Here, we discuss each mechanism and the role of astrocytes herein as acceptor cells of the prionoids.

- **Tunnelling nanotubes**

Neurons, and other cell types including astrocytes (Wang et al., 2011), have the ability to produce temporary and retractable F-actin-based tubular protrusions, called tunnelling nanotubes (TNTs) that allow direct communication between cells (Abounit et al., 2012). TNTs are different from filopodia and are involved in normal physiological functions in the cells, such as the transmission of electrical signals in neurons (Smith et al., 2011). The finely regulated formation of TNTs requires SNARE proteins that allow the membrane curvature and fusion (Abounit et al., 2012). The active transport of vesicles and organelles via TNTs (Rustom et al., 2004; Wang et al., 2015) is mediated by molecular motors (Abounit et al., 2012). Brain cells can form TNTs under stress conditions (for example during starvation and during pathological processes). It has been proposed that TNTs are formed as a defence mechanism to allow cells to expel material that cannot be degraded, such as proteinaceous aggregates in NDs (Victoria et al., 2017). On the other hand, the transfer of these aggregates can contribute to the pathological process. *In vitro* experiments have shown that prions and prionoids can induce the formation of and hijack TNTs in donor cells. TNTs become channels for the spreading of toxic aggregates to acceptor cell (Gousset et al., 2009). A-syn, for example, have been found to be efficiently transferred from donor to acceptor neurons through TNTs inside endo-lysosomal vesicles (Abounit et al., 2016).

Data in *in vitro* experiments with co-cultured neurons have shown that TNTs serve as channels also for the prion-like spreading of PolyQ HTT (Costanzo et al., 2013). PolyQ HTT overexpressed in donor neurons induces the formation of TNTs. When the nanotubes get in contact with the acceptor cells transport of PolyQ aggregates is enable. Differently from α-syn, PolyQ HTT aggregates observed in these experiments are not embedded in vesicles but rather encaged by vimentin in aggresomes-like structures (Costanzo et al., 2013), suggesting that PolyQ HTT prionoids spread via TNTs without the involvement of endo-lysosomal pathways. PolyQ HTT aggregates induce TNTs formation (Costanzo et al., 2013), but the exact mechanisms that regulate the formation of TNTs during HD and the transport of the PolyQ HTT aggregates through TNTs still need to be elucidated. Moreover, data showing that PolyQ HTT transfer via TNTs also occurs *in vivo* are missing.

Astrocytes can also form TNTs under stress conditions (Wang et al., 2011). Moreover, recent data have shown that α-syn can be transferred via TNTs between co-cultured astrocytes (Rostami et al., 2017). Although further investigations are needed to unravel the *in vivo* functional significance of these findings, one can hypothesize that PolyQ HTT aggregates may be transferred from neurons to astrocytes via TNTs. Astrocytes might temporarily serve as reservoir of such aggregates released via TNTs from the neighbour neurons, providing non-cell autonomous protection. Nonetheless, after a certain threshold, astrocytes might initiate to protrude TNTs toward neighbouring cells to expel the

toxic not degradable aggregates. One possible interpretation of our data could be that by potentiating their resistance against aggregate toxicity via overexpression of protective chaperones, such as DNAJB6, the reservoir capacity of astrocytes might be enhanced.

- **Endocytosis and phagocytosis**

Endocytosis mainly controls the internalization and recycling of plasma membrane components, receptors, and ligands and the uptake of extracellular macromolecules, nutrients and particles. The endocytic vesicles are formed at the plasma membrane and after internalization, they mature into early and late endosomes. These latter fuse with lysosomes for the degradation of the vesicle cargo.

Data from *in vitro* and *in vivo* studies have suggested that NDs-associated aggregates, such as α-syn (Sung et al., 2001; Park et al., 2009; Hansen et al., 2011; Valpolicelli et al., 2011; Angot et al., 2012; Konno et al., 2012; Oh et al., 2016) and tau (Frost et al., 2009; Guo et al., 2011; Wu et al., 2012), can enter in cells in a prion-like manner through different mechanisms of endocytosis (Costanzo et al., 2013).

Similarly, PolyQ HTT has been suggested to be internalized in cells through clathrin-dependent endocytosis (Ruiz-Alrandis 2016). PolyQ HTT exon-1 fibrils (HTTExon1Q$_{44}$) added to the culture medium of neuronal-like cells were found in early endosomes in the first hours after exposure; at later times, the same aggregates were found in late endosomes and lysosomes, suggesting that the acceptor cells direct the endosomal cargo toward a degradation pathway (Ruiz-Alrandis et al., 2016). These findings indicate that endocytosis might be linked to prion-like spreading of PolyQ HTT, but also that fibrils that were internalized in fact may be detoxified by lysosomal degradation. Importantly, the results of this study indeed indicate that fractions of PolyQ HTT fibrils escape the endo-membranous compartment before being degraded and reach the cytosol, where they can initiate seeding. Although the authors do not provide data, it is suggested that PolyQ aggregates may escape the endosome through leakage or rupture, as observed for α-syn (Freeman et al., 2013) and amyloid beta peptide (Ji et al., 2002). In astrocytes, extracellular particles may not only enter via such endocytotic routes but also through phagocytosis as suggested by Pearce and colleagues (Pearce et al., 2015).

Whilst uptake of aggregates by astrocytes could halt the speed of neuron-to-neuron transmission, a prerequisite would be that the astrocytes “survive” after having taken up this material. Internalized PolyQ HTT aggregates can escape the phago-lysosomal membrane and end up in the cytoplasm, where they induce co-aggregation of cytoplasmic normal HTT and co-localize with cytoplasmic chaperones such as HSP70/HSC70 and HSP90. If such escape occurs, aggregates might cause loss of astrocytes functionality and vitality with the consequent loss of their neuroprotective activity. As postulated before, and consistent with our findings, the clearance and reservoir capacities of these cells might be enhanced by potentiating their resistance against aggregate toxicity, via overexpression of protective chaperones, such as DNAJB6.

- **Exosomes and exophers**

Exosomes are extracellular vesicles (typically 40–100 nm) with a key role in intercellular communication and for the transmission of macromolecules between cells (including mRNA and proteins): they are formed by the fusion of the multivesicular body (an intermediate of endocytic compartment) with the plasma membrane, followed by the budding of the resulting vesicles, which are finally internalized by the acceptor cells (Edgar et al., 2016). Although the mechanisms underlying the selection and loading of the cargo, the budding, and the entrance in the acceptor cells still need to be completely elucidated, almost all mammalian cells with an endomembranes system are capable to release exosomes (Edgar et al., 2016).

It has been shown that NDs-associated aggregates can be also loaded in exosomes, secreted in the extracellular space and delivered to acceptor cells through these vesicles in a prion-like manner (Soria et al., 2017): similarly to TNTs, this might be a cellular strategy to expel non-degradable material that however contributes to the pathological spreading of toxic aggregates. The exact mechanisms by which aggregate species are loaded in exosomes and the destiny of the cargo, once internalized in the recipient cells, still need to be elucidated (Soria et al., 2017).

The subsequent uptake of exosomes by the recipient cell involves fusion with the plasma membrane and different cell-specific mechanism including endocytosis, phagocytosis (Feng et al., 2010, Fruhbeis et al., 2013; Abels and Breakefield, 2016) and macropinocytosis (Fitzner et al., 2011). Whether and how the acceptor cells select the exosomes with the cargo still need to be elucidated. Astrocytes can release and uptake exosomes to transmit many different types of cargos including mRNA, cytokines and peptides (Verkhratsky et al., 2016). Studies have shown that ND-associated aggregates, such as SOD-1, can be loaded and released as cargo in exosomes by astrocytes (Haidet et al., 2011; Basso et al., 2013). Investigations are needed to confirm whether PolyQ HTT aggregates are also loaded in astrocyte-derived exosomes.

Jeon and colleagues have shown that PolyQ HTT can be transferred between brain cells via exosomes *in vitro* and *in vivo* (Jeon et al., 2016). Interestingly this is the first study of human-to-mouse exosome-mediated transmission of toxic ND-associated aggregates. Co-culture of neurons derived from murine neural stem cells with human fibroblasts from an HD patient carrying a 143 CAG repeats mutation in *HTT* gene (HD143F) showed that mutant PolyQ HTT can spread in neurons in a prion-like manner, as cargo in exosomes from the donor fibroblasts. Also, the injection of exosomes derived from human HD143 fibroblasts in wild type mice triggered HD-like symptoms together with appearance of HTT aggregates in the animal brain. Data from this study also suggest that the release of PolyQ HTT in exosomes is not due to cell death and that the spreading of aggregates does not always require cell-to-cell contact (Jeon et al., 2016). Further investigations are needed to elucidate if the internalized Poly HTT aggregates are capable to escape from the endocytic pathway and to be vesicle-free in the cytosol.

More recently, Melentijevic and colleagues have shown that cellular stress and the presence of PolyQ HTT aggregates can induce in *C. elegans* the formation of vesicles named exophers (Melentijevic et al., 2017). They are capable to incorporate and extruding organelles and aggregates

and are thought to be a cellular defence mechanism that however contributes to the cell-to-cell transmission of toxic aggregates (Melentijevic et al., 2017).

Due to their key role in vesicle trafficking and on the base of data from these studies, we can speculate that astrocytes may be capable to uptake exosomes (or exophers), loaded with PolyQ HTT aggregates released from neighbouring cells and that this prion-reservoir activity can contribute to protect the brain. The later release of exosomes by astrocytes might be an attempt to expel the aggregates. These unconventional secretory pathways might be only triggered once the cells are not further capable to cope with the presence of these toxic species (Basso et al., 2011).

- **Direct penetration of the plasma membrane**

Intracellular and extracellular aggregate species, including PolyQ HTT (Bäuerlein et al., 2017), directly interact with the phospholipid bilayer of the membranes. Aggregates can cross the phospholipid bilayer through the formation of transmembrane channels (Arispe et al., 1993; Jang et al., 2010), membrane deformation or disruption (Reynolds et al., 2011).

Ren and colleagues have shown that synthetic PolyQ fibrils (PolyQ stretch flanked by lysine residues, $K_2Q_{44}K_2$), added to the culture medium, enter in various type of acceptor mammalian cells (Cos-7, CHO, HEK, HeLa, and N2A) (Ren et al., 2009). This suggests that various brain cells, including astrocytes, might be recipients for these prionoids. $K_2Q_{44}K_2$ fibrils directly spread into the cytoplasm of the recipient cells, where they co-aggregate with endogenous normal or PolyQ HTT and co-localize with cytosolic quality control components such as ubiquitin and HSP70. Using transmission electron microscopy, the authors showed the presence of fibrillar aggregates attached to the inner surface of the plasma membrane on a “bed” of cortical actin with no evidence that such aggregates were surrounded by endomembranous structures or clathrin (Ren et al., 2009). These data suggest that PolyQ species may directly penetrate into the plasma membrane and enter the cytosol. However, further research is needed to confirm this in co-culturing systems and *in vivo* experiments, and to unravel the mechanisms of membrane penetration. Importantly, data from the same group showed that PolyQ fibrils, different from those previously used (PolyQ HTT exon-1 fibrils, $HTTExon1Q_{44}$), enter the cells through endocytosis (Ruiz-Alrandis et al., 2016).

- **Transynaptic propagation**

PolyQ HTT aggregates have been found in axonal terminals and associated with synaptic vesicles (Li et al., 2003). In a study by Pecho-Vrieseling and colleagues, a possible pathological significance for this has been investigated using *ex vivo* neural network models, in which neurons from R6/2 HD mice establish synaptic contacts with neurons from wild type animals (or alternatively, neurons differentiated from human embryonic stem cells) (Pecho-Vrieseling et al.,2014). Spreading of PolyQ HTT from the R6/2 neurons to the wild type neurons was observed. PolyQ HTT was located between and in close proximity to the marker synaptophysin in the pre-synapses, and to PSD-95 marker in the post-synapses. Additionally, up-taken aggregates by recipient neurons were initially located in the cytoplasm, but subsequently in the nucleus. Finally, the PolyQ HTT spreading was blocked by botulinum neurotoxins, which are well known to block synaptic vesicle fusion and neurotransmission in the pre-synapses by targeting different SNARE proteins (Pecho-Vrieseling et

al., 2014). Taken together, these data suggest a synaptic mechanism underlying the transneuronal propagation of PolyQ HTT aggregates. However further investigations are needed, for example to elucidate how PolyQ HTT in the presynaptic neuron can reach the terminal axon.

Each synapse in the brain is monitored by astrocytes and it is estimated that the processes of one astrocyte can contact over 100 000 synapses. Astrocytes exert key functions at the multi-partite synapse: they have a fundamental role in the vesicles trafficking in the synaptic cleft, respond to synaptic neural activity, regulate the synaptic transmission and keep the homeostasis of fluid, ions, pH and neurotransmitters (Araque et al., 1999; Halassa et al., 2007; Perea et al., 2009). We can therefore hypothesize that astrocytes can also play an important role in the transynaptic propagation of PolyQ HTT aggregates. Promoting their capacity to uptake PolyQ-loaded vesicles from the synaptic cleft and boosting their resistance to PolyQ HTT toxicity could greatly contribute to slow down the neuron-to-neuron spreading.

1.3 - Intercellular transmission of chaperones from astrocytes to neurons and implications in Huntington's

Altough our data do not support it, a possible intriguing mechanism - that we have taken in consideration to explain the non-cell autonomous DNAJB6 protection in our HD model - is the intercellular transmission of the chaperone from astrocytes to neurons.

A recent study from Takeuchi and colleagues showed that chaperones of the HSP40/DNAJ, HSP70/HSPA and HSP90/HSPC families can be transported between cells via exosome release and uptake. Notably, they found that HSP40/DNAJ-containing exosomes added to the culture medium of acceptor cells expressing PolyQ HTT can efficiently reduce aggregate formation (Takeuchi et al., 2015). Interestingly DNAJA1, DNAJA2, and DNAJB6a (a longer isoform of DNAJB6, different from the shorter DNAJB6b used in our study) (Hageman et al., 2010, Hanai et al., 2003), have been found to be secreted in exosomes, whereas DNAJ proteins localized in organelles (such as DNAJA3 in mitochondria, Hageman et al., 2010) have not. Exosome secretion of HSP40 resulted to be dependent by the J domain in the chaperone (Takeuchi et al., 2015).

The cell-to-cell exchange of HSPs using exosomes has been proposed by Takeuchi and colleagues as a physiological mechanism to cope with the variable capacity of different cells to express chaperones under stress conditions: in this proposed model, cells with an intrinsic limited capacity to respond to proteotoxic stress receive HSPs-loaded exosomes from the more responsive cells (Takeuchi et al., 2015).

Although our data suggest that an exosome-secretion and uptake is not the dominant mechanism for the non-cell autonomous protection of astrocytic-DNAJB6 in our HD *D.melanogaster* model, the possible transport of protective chaperones between cells via exosomes still assigns a compelling physiological role of these vesicles in maintaining organismal protein homeostasis. In section 3.4.2 of Chapter 2, we discussed how brain cells have different capacities to induce the Heat Shock Response (HSR) (Sala et al., 2017; San Gil et al., 2017): astrocytes show a faster and stronger response than neurons (Nishimura et al., 1991) and whereas neurons in rodent *in vivo* models do

not induce HSPA/HSP70 expression after exposure to stress conditions, astrocytes do (Manzerra et al., 1992; Nishimura et al., 1996; Manzerra et al., 1997; Krueger et al., 1999; Oza et al., 2007; Pavlik et al., 2007; Yang et al., 2008). The lower intrinsic capacities to mount the HSR of neurons when compared to astrocytes might be functionally compensated by the intercellular astrocytes-to-neurons transmission of exosomes loaded with HSPs.

Takeuchi and colleagues also suggest that an eventual enhancement of the secretion of HSP-loaded exosomes might be a potential therapeutic strategy for HD and other neurodegenerative diseases (NDs) characterized by protein aggregation (Takeuchi et al., 2015). Although this indeed might be a potential strategy to promote the protein homeostasis in the brain affected by NDs (for example by stimulating astrocytes to express and release exosomes loaded of protective chaperones towards the surrounding neurons), this eventual therapy should aim to allow the exclusive loading of HSPs as cargo in the exosomes in the donor cells. This is a critical aspect in consideration of the fact that exosomes have been also found as vehicle of disease-associated prionoids such as α-syn (Emmanouilidou et al., 2010), Aβ (Rajendran et al., 2006), tau (Saman et al., 2012), SOD-1 (Gomes et al., 2007) and PolyQ-HTT (Jeon et al., 2016). The mechanisms underlying the selection of the exosome cargo during the pathology must be elucidated to develop potential therapies based on this strategy.

Exosomes are however ideal delivery vectors due to their low immunogenicity and toxicity and capacity to cross membranes (El Andaloussi et al., 2013). Recently, methods to produce engineered exosomes loaded with a specific protein have been developed. Exosomes engineered in laboratory have been successfully administered to the brain of mice, via the nasal route, proving their potential for the *in vivo* delivering of selected proteins (Sterzenbach et al., 2017). These findings open to future therapeutical approaches in which protective HSPs (such as DNAJB6) in exosomes are delivered to the brain for the treatment of aggregate-associated NDs.

1.4 - Protection by DNAJB6: cell autonomous and non-cell autonomous mechanims

Our data showed that the cytosolic isoform DNAJB6b (Hanai et al., 2003) is an interesting candidate chaperone to enhance both the cell intrinsic resistance to polyQ aggregation (Chapter 4) as well as to boost the non-cell autonomous protection of astrocytes against HD pathogenesis (Chapter 5).

What is known about the cell-autonomous protective activity of DNAJB6 against PolyQ Htt aggregation and aggregate toxicity?

The formation of PolyQ Htt aggregates is mechanistically described as a process in different steps that starts with the formation of an initial “nucleus”, a kinetically unstable oligomer formed from interacting monomers (primary nucleation). Once the nucleus is assembled, it is thought to grow into large and highly stable β-sheet-rich structures with a fibrillary morphology. The growth of the fibrils proceeds through monomer addition on the existing β-sheet-rich aggregates (Morris et al., 2009; Cohen et al., 2011; Cohen et al., 2012). The aggregation process proceeds with secondary processes such as the fragmentation of the fibrils (Xue et al., 2008; Knowles et al., 2009), and the

nucleation at the surface and ends of the existing fibre (elongation sites) (Ferrone et al., 1985; Ruschak et al., 2007; Cohen et al., 2013) and fibril branching (secondary nucleation) (Andersen et al., 2009). A large amount of data indicates that the formation of PolyQ Htt aggregates occurs through these processes (Scherzinger et al., 1997; Scherzinger et al., 1999; Chen et al., 2002; Poirier et al., 2002; Dahlgren et al., 2005; Thakur et al., 2009; Crick et al., 2013; Wagner et al., 2018). The presence of the PolyQ expansion is thought to be the main driver of aggregation of PolyQ Htt, but other factors might influence its fibrillo-genesis, including the state of the PolyQ Htt monomers forming the aggregates (i.e. full length protein or truncated Htt fragments derived by proteases cleavage), the length of the PolyQ expansion, the protein concentration and the time.

DNAJB6 is a HSP70 co-chaperone that reduces the PolyQ-induced toxicity *in vitro* and *in vivo* (Hageman et al., 2010; Kakkar et al., 2016 and our data). The protective mechanism of action of the chaperone mainly relies in its capacity to form oligomeric complexes and to directly interact with the PolyQ client (Hageman et al., 2010). DNAJB6 strongly inhibits the primary nucleation step and perturbs the secondary nucleation in the aggregation process (Kakkar et al., 2016), by interaction of a serine/threonine (S/T)-rich region in its C-terminus domain with the substrate (Kakkar et al., 2016; Söderberg et al., 2018). The current model postulates that the hydroxyl groups in the side chains of the S/T region of DNAJB6 reduce the nucleation rate of the PolyQ species by competing with the hydrogen bonding necessary for formation of amyloid fibrils and beta-hairpins (Hoop et al., 2016).

However, little is know about the fate of the PolyQ Htt-DNAJB6 complex. Although not absolutely required for the anti-aggregation activity (Hageman et al., 2010), the J-domain of DNAJB6 allows the interaction with HSP70/HSPA that might direct the PolyQ species for further processing. Data concerning the physiological role of DNAJB6 (Watson 2007, Izawa 2000) or about chaperonopathies linked to to mutations of the *DNAJB6* gene (Harms et al., 2012; Sarparanta et al., 2012; Suarez-Cedeno et al.,2014) might provide insights about the possible processing of the PolyQ Htt-DNAJB6 complex.
In our proposed model (Chapter 5, Figure 6), we speculate that somehow the DNAJB6-expressing astrocytes become a more effective reservoir of PolyQ HTT prionoids (which might be up-taken through different mechanisms such as TNTs-mediated transmission, uptake of exosomes and transynaptic vesicles or by endocytosis) and slow down the pathological spreading, as described above.

But the unanswered question remains: how can DNAJB6 make astrocytes more resistant to the uptaken PolyQ Htt prionoids, therefore maintaining their capacity to act as “prion-reservoir” and counteract the cell-to-cell spreading?

As said before, PolyQ Htt prionoids, independently from the mechanisms of entry, can end up in the cytosol as vesicle-free aggregate species with strong seeding properties and capable to interact with endogenous proteins and organelles. Interestingly, the isoform DNAJB6b (Hanai et al., 2003) is cytosolic (and nuclear) allowing interaction with these PolyQ species. Hence, based on the knowledge on PolyQ Htt prionoids and DNAJB6, we can speculate the following possible (mutually non-exclusive) mechanisms underlying the protective activity of astrocytic DNAJB6:

1. Shielding/capping of PolyQ Htt prionoids and slowing down of seeding processes

DNAJB6 may bind to fiber ends, hereby such shielding/capping of the prionoids might slow down their growth into (more) toxic amyloid fibrils. Even though DNAJB6 was found to be most effective in preventing primary nucleations, DNAJB6 has been shown to be still quite effective in suppressing secondary nucleations (Kakkar et al., 2016). Yet, data from our lab showed that the seeding of endogeneously expressed soluble non-expanded polyQs proteins by extracellular addition of polyQ fibrils (Ren et al., 2009) was only marginally affected by DNAJB6 (Kakkar et al., 2016). PolyQ Htt prionoids provide surfaces for events of seeding with cellular proteins (including normal Htt) (Kazantsev et al. 1999, Busch et al. 2003; Ren et al. 2009; Trevino et al. 2012; Holmes et al. 2013; Tan et al. 2015; Ruiz-Alrandis et al. 2016), and - as well as endogenous aggregates - they can interact with organelles and cytoskeleton (Chapter 2, section 2.4). The DNAJB6 shielding/capping might impede the seeding and the interaction of prionoids with these cellular elements in the astrocytes. By slowing down all the cytotoxic events derived by seeding and interaction, DNAJB6 might provide a protective effect against the PolyQ Htt prionoids in the astrocytes. Alternatively, DNAJB6 binding of the fibrils may lead to active detoxification mechanisms as described below.

2. (De-)acetylation of PolyQ prionoids for processing

DNAJB6 can interact with several histone deacetylases (HDAC), including HDAC4 and HDAC6 (Hageman et al., 2010). HDAC4 is required for the full anti-aggregation activity of DNAJB6 and inhibition of HDAC4 results in a loss of function of DNAJB6 (Hageman et al., 2010). The acetylation state of DNAJB6 and PolyQ Htt might therefore be crucial in the mechanism. Hence, we can speculate that PolyQ Htt prionoids in astrocytes shielded by DNAJB6 are subjected to HDAC-mediated (de)acetylation. Interestingly, previous studies showed that HDACs are involved in protein quality control (Pandey et al., 2007; Jeong et al., 2009). The acetylation mediated by DNAJB6 and HDAC4 in astrocytes might direct the prionoids towards some of the pathways described below, or promote their compartmentalisation in specific cell sites. In other words, the acetylation of the prionoids mediated by DNAJB6/HDAC recognition might be the starting point for detoxification processing as described below.

3. Promoting the sequestration in cellular deposit sites

Cells can sequester misfolded or aggregated proteins into specific cellular deposit sites to prevent their toxicity as a key strategy of defense against protein aggregation (Tyedmers et al., 2010). Aggresomes in mammalian cells are a transient form of regulated aggregate deposition (Johnston et al., 1998; Kopito et al., 2000; Garcia-Mata et al., 2002): their formation and movement along the microtubules of the cytoskeleton (Garcia-Mata et al., 1999) is mediated by the activity of the motor protein dynein and adaptor proteins, like histone deacetylase 6 (HDAC6) (Kawaguchi et al., 2003). Such sequestration may serve to transiently store the cargo, for further refolding (Nollen et al., 2001) or degradation (Park et al., 2013). Hageman and colleagues found that DNAJB6 can interact with HDAC6 (Hageman et al., 2010). We might therefore speculate that DNAJB6 in astrocytes recognizes and binds the PolyQ Htt prionoids and through its interaction with HDAC6, direct the prionoids to these cellular deposition sites. In support of this, recent data have shown that DNAJB6, together with HSP70, is involved in protein sequestration in yeast (Kumar et al., 2018). The sequestration of PolyQ prionoids in a cellular deposit site, mediated by DNAB6, might be an effective

protective strategy in astrocytes also because, differently from post-mitotic neurons, astrocytes can divide. As last resort, astrocytes might asymmetrically partitionate the sequestered prionoids and benefit one of the daughter cells with a lower aggregate load ("dilution effect"; Rujano et al., 2006; Fuentealba et al., 2008). The survived astrocytes might then continue to support the neuronal fitness and continuing in the uptake of other spreading prionoids.

4. **Disaggregation processing**

Disaggregation is characterized by the recognition of the aggregate by sets of HSPs that actively participate to the one-by-one extraction of misfolded polypeptides (Mogk et al., 2018). Disaggregation in mammalian cells is mainly mediated by HSPA/HSP70, assisted by specific set of co-chaperones - members of the HSP110 family and DNAJs (e.g. DNAJA2 and DNAJB1 in humans) (Nillegoda et al., 2015; Mogk et al., 2018) - that empowers HSPA/HSP70 to exhibit a potent, standalone disaggregation activity. Small HSPs (sHSPs) are thought to facilitate this "extraction" process by binding the aggregating substrates and changing the structure of the aggregates such that they remain in a (more) disaggregation-competent form (Nillegoda et al., 2015; Mogk et al., 2018). Although data from our lab showed that DNAJB6 is not *per se* capable to disaggregate PolyQ aggregates (Hageman et al., 2010), we might hypothesize that DNAJB6-capping of PolyQ Htt prionoids might still promote their recruitment in the disaggregation processing pathway. Similarly to sHPS, DNAJB6 might be capable to keep the PolyQ substrate in a disaggregation-competent form. DNAJB6 might then recruit HSPA via its J domain directing the prionoid toward a disaggregation processing.

5. **Re-engagement towards the autophagic flux or proteasomal degradation**

The J-domain allows to DNAJB6 to recruit HSPA, providing a link to direct the PolyQ Htt prionoids towards protein degradation pathways, such as autophagy and proteasome degradation. This might occur right after acetylation and disaggregation of the DNAJB6-capped PolyQ substrate. Previous data already suggest that DNAJB6 is involved in protein degradation and catabolic processes (Izawa et al., 2000; Watson et al., 2007, Kakkar et al., 2016). Although PolyQ Htt is known to be a poor substrate for these proteolytic pathways (Ciechanover et al., 2015), they might represent a line of defence against the toxicity of the prionoids in the astrocytes.

Future studies might explore how DNAJB6 is capable to protect astrocytes by starting from the above hypothesis. Data would provide further insights in the mechanisms of protection of DNAJB6 against PolyQ Htt, but also about the processing of toxic prionoids by astrocytes and how this influences the course of the pathology.

All the previous mechanisms relies on the idea that PolyQ Htt prionoids enter in astrocytes where they interact with DNAJB6. Would it be possible instead that DNAJB6 is first released by astrocytes and next uptaken by the suffering neurons expressing PolyQ Htt? In the next section, I will discuss this interesting option.

1.5 - Other glial cells might come into play

Our data showed that astrocytes are key players in the non-cell autonomous protection mediated by DNAJB6 against PolyQ Htt. Although our data suggest that other glial cells seem not crucial in the DNAJB6-related non-cell autonomous protection - emphasizing the pivotal role of astrocytes in the maintainance of brain homeostasis - still we cannot exclude the participation of other glial cell types, such as microglia in the protective mechanisms as described above. Microglia and astrocytes establish several functional interplays during brain disease, therefore an important line of research would be the investigation of how the modulation of the chaperonome in astrocytes influences such astrocyte-microglia interplay in HD.

Microglia are the resident macrophages in the central nervous system, they monitor the environment and respond to neural degeneration by switching to different activation states. Similarly to astrocytes (Section 3 of Chapter 2), microglial activaction might play a dual role in HD either being neuroprotective or detrimental for neurons. They are fundamental actors in the mechanism of neuroinflammation because involved in the release of inflammatory cytokines and clearing of cell debris (Ywang et al., 2017). Microglial activaction is a component of the pathogenesis of HD and it is believed to be triggered by PolyQ Htt-mediated cytotoxicity (Ywang et al., 2017). Accumulating evidences show that PolyQ Htt can trigger microglial activation (Crotti et al., 2015). Importantly, microglia hardly show PolyQ Htt aggregates in different HD rodent models and brains of HD patients (Jansen et al., 2017). Such less frequent presence of aggregates in microglia compared to neurons might be due to differences in protein quality control, and expression/activity of HSPs.

A cross-talk between astrocytes and microglia occurs during HD pathology, meaning that both the cell populations release molecules and other signals that are crucial for the regulation of their cellular activities (Crotti et al., 2015; Ywang et al., 2017). Such crosstalk is also important for the modulation of the processes of neuroinflammation (Crotti et al., 2015).

The interplay between astrocytes and microglia offers new perspectives to our findings. Astrocytes with potentiated chaperonome might be capable through the release of specific signals to modulate the microglia activaction and neuroinflammation, maintaining the microglia state toward a neuroprotective path (e.g. control of cytokine release). The DNAJB6-potentiated astrocytes and microglia might also efficiently collaborate in limitating the spreading of PolyQ Htt prionoids.

One intriguing possibility would be to investigate the effects in HD pathology of the expression of protective chaperones in microglia. Given the crucial role of both glial cell types in maintainig neuronal homeostasis, a combined expression of protective chaperones in astrocytes and microglia might offer an additional layer of protection against PolyQ Htt aggregates.

2. Saving neurons is good, together with astrocytes is better: the "healthy-astrocytes" approach

The development of treatments of neurodegenerative diseases - a field that in the last decades showed several important advances in the understanding of the pathology, but with very limited success in finding a therapy - should be based on evidences that go beyond an exclusive neuron-centric view and that consider more wide aspects such as the strict functional interdependency between neurons and astrocytes (and other glial cells). Neurons are the "kingmakers" of the brain physiology and pathology, but an increasing amount of evidences is showing that NDs are diseases of neurons with a key co-participation of astrocytes and other glial cells: if neurons are sick, also astrocytes are, and vice versa.

An increasing amount of evidences, including our findings indicate that targeting the prion-like spreading of PolyQ HTT prionoids in order to slow down the pathological processes in HD might have a therapeutical value.

Although further investigations are needed to confirm whether and how the spreading occurs *in vivo,* the data from the above suggest the following key points:

1. The mechanisms of spreading of PolyQ HTT prionoids, which involve TNTs communication, exosomes / exophers release, and transynaptic propagation, are not unregulated passive events due to the death of the cells. Rather, they are active and well-regulated processes that the donor cells use when they are not further capable to cope with the presence of these toxic species, used as mechanisms of defense in the early phase of the pathological process. Importantly, these mechanisms result to be less active and efficient when the disease is progressing and the cells are degenerating. If from one side, these mechanisms can temporarily protect the donor cell, from the other side, they can promote the spreading of the prionoids to the recipient cells.
2. Cell-to-cell contact seems an important factor to promote the spreading of PolyQ HTT, but it is not always required.
3. HTT prionoids can actively enter the cells embedded in vesicles or vesicles-free, and in a regulated manner (for example, by exosome uptake or by clathrin dependent endocytosis). However other mechanisms can also occur, such as passive membrane penetration or phagocytosis. The mechanism of uptake mainly depends by the types of acceptor cells, the aggregate species and disease stage.
4. Differently from other prionoids, PolyQ HTT prionoids, independently from the mechanisms of entry in the recipient cells, can end up in the cytosol. Here, these vesicle-free aggregate species have seeding properties and can interact with endogenous proteins (including components of the protein quality control, such as cytosolic HSPs). Further investigations are needed to understand if this occurs by escaping of the PolyQ HTT aggregates from the endocytic-lysosomal compartment.

Taking in consideration the above, one can speculate that by inhibiting the mechanisms involved in the spreading of PolyQ HTT prionoids, it would be possible to slow down the disease progression of HD. However, there is a number of limitations to consider about this approach:

- Some of these mechanisms such as the exosome release and the TNTs-mediated communication are used by the donor cells as defense strategies against the aggregate toxicity. A complete blockage of the spreading could result in an acceleration of the pathological process in the most vulnerable cells (likely neurons). Therefore, the magnitude by which the spreading should be inhibited and the specific cells to target are factors that must be taken in consideration to design an effective therapy.
- The inhibition of some of these mechanisms could have detrimental consequences for the cell functionality and viability. Some of them, such as transynaptic communication, endocytosis, and TNTs communication can be blocked by a pharmacological treatment (for example by using respectively botulinum neurotoxins, chlorpromazine or cytochalasin B) (Ruiz-Alrandis et al., 2016; Pecho-Vrieseling et al., 2014; Bukoreshtliev et al., 2009). However, we must consider that they also are key fundamental cellular processes that are important for the transport of molecules, nutrients, neurotransmitters and more, and do not involve only the spreading of PolyQ HTT prionoids. Moreover, many pharmacological inhibitors might not target the selected pathway in a specific manner, and therefore produce side-effects, due to off-target inhibitions.
- The blockage of the spreading of PolyQ HTT aggregates might likely not suffice to efficiently slow down the pathology as the formation of the aggregates still occurs in a cell-autonomous manner (Walsh et al., 2016).

Here we speculate that a “healthy astrocytes” approach against toxic PolyQ HTT prionoids based on the overexpression of the protective chaperone, such as DNAJB6, in the astrocytes (as investigated in our study), might slow down the pathological processes and overcome some of the previous described limitations and side effects.

The combined strategies to provide cell-autonomous and non-cell autonomous protection by expressing DNAJB6 in different types of brain cells - neurons or astrocytes respectively - might be therapeutically very effective. DNAJB6 is expressed ubiquitously (Hageman et al., 2010) and *in vivo* brain-specific overexpression of DNAJB6 is not associated with deleterious phenotypic effects (Kakkar et al., 2016): these findings support the idea that DNAJB6 might be a safe therapeutic target in HD and that its expression might be promoted in both neurons and astrocytes.

As shown by our findings, such approach also provides new insights about the functional role of chaperones in the brain during the pathology of PolyQ diseases (when these chaperones are expressed in the different cells of the nervous tissue). The non-cell autonomous protection via HSP expression in astrocytes highlights the unique role of these glial cells in supporting and protecting neurons and paves the way towards new therapeutic strategies against HD.

In more general terms, we can conclude that the potentiation of the brain cell chaperonome might have a great therapeutical value in neurodegenerative diseases. By overexpressing the chaperone in neurons and stopping the protein aggregation, we aim to directly protect these cells. However, this approach may be scaled-up and improved by also boosting the chaperonome of astrocytes: in this case, we do not only provide protection in a cell-autonomous manner, but also in a non-cell autonomous manner, promoting the capacity of astrocytes to keep the neuronal viability and fitness.

In conclusion, astrocytes can indeed be an important target of treatment in neurodegenerative diseases. Paraphrasing the words of Prof. Ben Barres in a 2008 perspective article, many drug trials exclusively target neurons, whereas the role of astrocytes and other glial cells in the pathological process still has received little attention. If the astrocytes that physiologically support neurons are killed, "*how can the neurons be saved by just targeting the neurons?*" (Barres, 2008).

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Samenvatting

Neurodegeneratieve ziekten zoals de ziekte van Huntington, Alzheimer en Parkinson beïnvloeden het centrale zenuwstelsel en leiden tot degeneratie van een specifieke populatie neuronen in, voor elke ziekte andere, specifieke hersengebieden. De degeneratie van deze neuronen leidt vervolgens tot een progressief falen van die ziekte-specifieke hersengebieden en bepaalt het verschillende spectrum van symptomen bij de patiënten, zoals geheugenverlies bij de ziekte van Alzheimer en de bewegingsdefecten (chorea) bij de ziekte van Huntington. Vooral omdat de gemiddelde levensverwachting is toegenomen neemt het aantal mensen met neurodegeneratieve ziektes ook sterk toe. De huidige behandeling is vooral gericht op vermindering van (last van) de symptomen (gevolgen) en er zijn nog steeds geen therapieën die de oorzaak van deze ziekten kunnen wegnemen en tot voorkoming of genezing leiden.

Hoewel al deze ziekten verschillende klinische kenmerken hebben is er een belangrijk kenmerk dat ze delen in de hersenen van alle overleden patiënten: we vinden eiwitaggregaten, een onnatuurlijk klontering van eiwitten, in het gedegenereerde deel van het hersenweefsel. Hoewel we nog niet precies weten hoe (en welk type) aggregaten neurotoxisch zijn, is op diverse manieren al wel aangetoond dat het proces van eiwitaggregatie neurodegeneratie veroorzaakt.

Het ontstaan van eiwitaggregaten is op zich merkwaardig omdat de cellen in ons lichaam een heel uitgebreid verdedigingssysteem hebben die ze tegen het proces van eiwitaggregatie beschermd: dit systeem wordt het “protein quality control” (PQC) systeem genoemd. In dit PQC netwerk is een centrale rol weggelegd voor eiwitten uit een grote familie van zogenaamde “heat shock proteins” (HSP’s) Deze HSP's (ook vaak chaperonnes genoemd) herkennen niet- of verkeerd gevouwen eiwitten. Er zijn verschillende HSP’s families waaronder de familie van HSPA's (13 leden), DNAJ's (50 leden) en kleine HSP's (10 leden). Ze zijn vooral bekend van het helpen bij het vouwen van eiwitten tijdens hun synthese, of bij hun hervouwing als ze zijn ontvouwen na stress. Ze zijn echter bij veel meer processen betrokken waaronder het uitelkaar halen van eiwitklonten (eiwitdisaggregatie), het opslaan van ontvouwen eiwitten in depots, maar ook bij de eiwitafbraak, een proces dat vervolgens wordt uitgevoerd door het ubiquitine-proteasoomsysteem (UPS: de eiwitversnipperaar) dat plaatsvindt in het lysosoom (de cellulaire verbrandingsoven).

Naast eiwitaggregatie is een ander belangrijk gemeenschappelijk kenmerk bij deze hersenziekten de aanwezigheid van en verandering in niet-neuronale afweercellen - de glia cellen- in de aangedane hersengebieden. Met name de zogenaamde astrocyten - een grote en specifieke subpopulatie van glia cellen - veranderen van vorm en laten een groot aantal moleculaire veranderingen zien, waaronder een verhoogde aanwezigheid van bepaalde leden van de diverse HSP’s families.

Het doel van dit proefschrift, uitgelegd in **hoofdstuk 1**, was om te onderzoeken of en hoe specifieke HSP’s gebruikt zouden kunnen worden om neuronen te beschermen tegen eiwitaggregatie en degeneratie. In het bijzonder wilden we weten of het kon en moest via een directe manier, d.w.z. door deze HSP in de neuronen zelf in te brengen (zogenaamde cel-autonome bescherming) of dat het ook mogelijk was op een indirecte manier door de HSP in te brengen in de astrocyten (zogenaamde niet-cel-autonome bescherming).

In **hoofdstuk 2** wordt vervolgens eerst de literatuur samengevat over hoe eiwitaggregatie neurodegeneratie kan veroorzaken. Dit wordt o.a. ondersteund door het feit dat genetische vormen van deze neurodegeneratieve ziekten vrijwel altijd worden gekarakteriseerd door mutaties in de ziekte gerelateerde genen die leiden tot expressie van eiwitten die verhoogd aggregatiegevoelig zijn. In dit hoofdstuk wordt vervolgens samengevat hoe het aggregatieproces en de verschillende vormen van aggregaten veel processen in de cel kunnen verstoren. Verstoringen die zijn gevonden omvatten o.a. veranderingen in transcriptie, afwijkingen in het RNA-metabolisme, veranderingen in de calcium homeostase, interferentie met diverse onderdelen van het PQC-netwerk (HSP's, UPS en autofagie), fysieke schade aan organellen (bijv. ER, Golgi, mitochondria), het cytoskelet en de membranen en -vooral belangrijk voor neuronen- remming van axonaal transport en synapsfunctie. Belangrijk is ook dat neuronen erg kwetsbaar lijken te zijn voor eiwitaggregatie. In hoofdstuk 2 wordt vervolgens ingegaan op de steeds toenemende aanwijzingen dat aggregaten zich door de hersenen verspreiden op een 'prion-achtige manier' (van de gekke-koeien ziekte) en dat ziekteprogressie is geassocieerd met de overdracht van pathogene aggregaten tussen cellen. Vervolgens leggen we in hoofdstuk 2 uit hoe de diverse onderdelen van het PQC-netwerk in cellen de eiwithomeostase handhaven en hoe deze onderdelen mogelijk gebruikt kunnen worden bij het voorkomen of behandelen van deze eiwit aggregatie ziekten. Ten slotte benadrukken we hoe er een toenemend aantal aanwijzingen zijn gevonden die laten zien dat astrocyten niet alleen een aantal fundamentele fysiologische functies in de gezonde hersenen uitoefenen, maar hoe ze ook een mogelijke sleutelrol spelen in de pathogenese van neurodegeneratieve ziekten. Na neuronale schade worden astrocyten "hyper-reactief" en blijken allerlei moleculaire en functionele veranderingen te ondergaan. Initieel lijkt deze astrocyt-reactiviteit de progressie van de ziekte tegen te gaan, maar bij een langdurige reactiviteit lijkt dit juist negatieve effecten te hebben, die de progressie van neuronale degeneratie mogelijk zelfs kan versnellen.

In dit proefschrift richten we ons op de mogelijke beschermende werking van deze astrocyten, hoe ze mogelijk een rol kunnen spelen bij een remming van de prion-achtige verspreiding van aggregaten tussen neuronen, en hoe expressie van HSP's in astrocyten een dergelijke beschermende functie zou kunnen versterken. Om deze hypothese te testen, werd Drosophila melanogaster als modelsysteem gebruikt. Onder onderzoeksvraag vereiste dat we een systeem moesten ontwikkelen, waarbij het mutante PolyQ-huntingtine uitsluitend tot expressie zou worden gebracht in neuronen en een HSP, ofwel alleen in dezelfde neuronen of alleen in astrocyten. Als HSP kozen we DNAJB6 - een lid van de menselijke DNAJ-chaperonne familie - omdat ons laboratorium eerder had aangetoond dat dit DNAJB6 eiwit een zeer krachtige cel-autonome beschermer was tegen PolyQ-gerelateerde neurodegeneratie en omdat DNAJB6 ook verhoogd aanwezig bleek te zijn in de hyper-actieve astrocyten in sommige neurodegeneratieve ziekten.

In **hoofdstuk 3** beschrijven we hoe het D.melanogaster-model voor onze studie is gegenereerd. We laten zien hoe de benodigde specificiteit van de expressiesystemen werd verkregen die nodig was om selectieve expressie van alle kandidaatgenen te verzekeren ten behoeve van de evaluatie van cel-autonome en niet-cel-autonome effecten. Een reguleerbare expressie van de verschillende transgenen in de verschillende celtypen van de hersenen werd verkregen door het gebruiken van verschillende, zogenaamde binaire expressiesystemen - Gal4-UAS en LexA-LexO - en van goed gekarakteriseerde cel-specifieke promotors (ook drivers genoemd). Hierdoor konden we, op een volledig onafhankelijke en niet-overlappende manier, het PolyQ-

huntingtine uitsluitend tot expressie brengen in neuronen en DNAJB6 - de beschermende chaperonne - gelijktijdig tot expressie wordt brengen in dezelfde neuronen of alleen in astrocyten. Bovendien laten we zien dat het expressiesysteem LexA-LexO het inderdaad mogelijk maakt om het expressieniveau - gematigd of sterker - van het transgen te regelen.

In **hoofdstuk 4** hebben we eerst de cel-autonome effecten van de chaperonne DNAJB6 onderzocht. Hiertoe werden de D.melanogaster-modellen gebruikt die het mutante toxische PolyQ-eiwit en DNAJB6 uitsluitend in dezelfde neuronen tot expressie brengen. We laten zien dat DNAJB6 PolyQ-aggregatie in neuronen onderdrukt en beschermt tegen toxiciteit. De expressie en aggregatie van PolyQ verkortte de levensduur van de vliegen en overexpressie van DNAJB6 in de getroffen neuronen resulteerde ook in een significante verlenging van deze verkorte levensduur en verbeterde de fitness van de vliegen. Hierbij was de mate van bescherming afhankelijk van het expressieniveau van DNAJB6. Deze bevindingen bevestigden onze eerdere bevindingen dat DNAJB6 een sterke en directe beschermende werking heeft die PolyQ-eiwitaggregatie verlaagt en toxiciteit ervan vermindert.

In **hoofdstuk 5** hebben we hetzelfde D.melanogaster-model gebruikt om niet-cel autonome DNAJB6-effecten te onderzoeken. In dit geval werd, met het binaire expressie systeem, het toxische polyQ-eiwit selectief in neuronen en tegelijkertijd DNAJB6 selectief in astrocyten tot expressie gebracht. We ontdekten dat deze exclusieve expressie van DNAJB6 in astrocyten een niet-cel autonome bescherming biedt tegen de progressieve neuronale degeneratie en de levensduur van de vliegen met neuronaal PolyQ-huntingtine. Ook in dit geval was de bescherming gecorreleerd met de hoogte van de expressie van de DNAJB6. Anders dan wat we vonden toen de chaperonne tot co-expressie werd gebracht in neuronen (hoofdstuk 4), ging de niet-cel autonome bescherming niet gepaard met een vermindering van de polyQ-huntingtine-aggregatie in de hersenen van de vliegen. Wat we daarentegen vonden was dat, in vliegen die DNAJB6 in astrocyten tot expressie brengen, een groot deel van de astrocyten nu neuronale PolyQ-HTT-aggregaten bevatten. Dit is in lijn met de suggestie dat aggregaten uit degenererende neuronen zich kunnen verspreiden naar andere neuronen, die vervolgens daardoor ook zouden degenereren. Onze data suggereren dat, door deze extracellulaire huntingtine-aggregaten op te nemen - een capaciteit die wordt versterkt door DNAJB6-expressie- astrocyten kunnen fungeren als een "reservoir" voor deze prion-achtige aggregaten en daarmee verspreding van neuron naar neuron in de hersenen kunnen voorkomen en dus de progressie van neurodegeneratie vertragen. Normaliter wordt de capaciteit van astrocyten om als “prion-reservoir” te dienen waarschijnlijk beperkt door de toxiciteit van aggregaten die de cel opneemt. Onze data suggereren dat de verhoogde expressie van chaperonnes zoals DNAJB6 de toxiciteit van deze PolyQ aggregaten in astrocyten kan verminderen waardoor hun beschermende werking bij de ziekte van Huntington wordt versterkt.

In **hoofdstuk 6** vatten we onze bevindingen samen, bespreken hun relevantie in de context van andere bestaande literatuur gegevens, open vragen en geven we een overzicht van toekomstige onderzoeksperspectieven. Het is bijvoorbeeld nog onduidelijk welke mechanismen verantwoordelijk zijn voor de overdracht van PolyQ-prionachtige aggregaten van neuronen naar astrocyten en wat de mogelijke mechanismen waarmee DNAJB6 astrocyten resistenter kan maken tegen de opgenomen PolyQ Htt-prionoïden, waardoor ze hun capaciteit behouden om te fungeren als "prion-reservoir".

De cel-autonome bescherming van DNAJB6 lijkt effectiever dan de niet-cel-autonome bescherming. Terwijl dit sterk de gedachte ondersteunt dat PolyQ-ziekten primair neuronale ziekten zijn laten onze gegevens desalniettemin toch ook zien dat modulatie van het chaperonnes in astrocyten ook neuronale bescherming kan bieden, iets wat technisch wellicht minder complex zou kunnen zijn dan modulatie van chaperonnes in neuronen. Zeer waarschijnlijk zou een combinatie hiervan, verbeteren van PQC in zowel astrocyten als neuronen, optimaal zijn.

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